Cancer Stem Cells - Targeted Library

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1. Cancer Stem Cell Concept

Cancer stem cells (CSCs) are a specific subpopulation of tumor cells that selectively possess tumor initiation, pronounced self-renewal capacity and pluripotency as well as the ability to give rise to bulk populations of non-tumorigenic cancer cell progeny through differentiation [1]. Research over the last decade has highlighted a crucial role of CSCs in tumor genesis, growth and progression. They have been prospectively identified in several human malignancies, and their relative abundance in clinical cancer specimens has been correlated with malignant disease progression in human patients. Many scientists believe that cancers arise from CSCs, although this is currently under debate. The cancer stem cell hypothesis postulates that cancer can be initiated, sustained and maintained by CSCs rather than other stimuli. According to this hypothesis, CSCs are responsible for the occurrence of distant metastases and for tumor recurrence after initial effective treatment (reviewed in [2]). Currently, there are two key hypothetical explanations for the existence of CSCs. CSCs may arise from normal stem cells by mutation of genes that render the stem cells cancerous. Or, they may come from differentiated tumor cells that experience further genetic alterations and, therefore, become dedifferentiated and acquire CSC-like features. Indeed, it has been reasonably suggested that numerous factors may play a particular role in CSCs formation and development, including mutations, genetic predisposition, viruses and several external harmful exposures. For example, a wide number of retroviruses including HBV, AEV, FLV, M-MuLV and HTLV-1 have the ability to infect innate stem and progenitor cells, resulting in the deregulation of normal cell differentiation and the development of cancer. Other hypotheses that address the question of which target cell in cancer undergoes malignant transformation have also been suggested. Therefore, ongoing research in some of these models may provide a better understanding of intracellular processes leading to CSCs formation. It should be noted that the CSCs concept is "a relatively old idea reemerging at an important time" [3]. Recent evidence shows that normal stem cells may contribute to cancer development and progression by acting as cancer-initiating cells through their interactions with abnormal environmental elements. Normal stem cells and cancer stem cells (CSC) possess similar mechanisms of self-renewal and differentiation. CSC can be the key to the elaboration of anti-cancer-based therapy [4]. Currently, there is little knowledge about the cellular and

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molecular mechanisms that govern the initiation and maintenance of CSC. Studies on co-evolution and interdependence of cancer with normal tissues may result in novel therapies for treating cancers [5]. The involvement of CSCs in the formation of distant metastases, tumor dormancy and therapy resistance offers high hopes for treating cancer patients. Evidence [6] suggests a shared genomic fingerprint between embryonic stem cells, cancer cells, and cancer stem cells. The identification of CSCs has important implications for designing new therapeutic approaches for the treatment and prevention of cancer.

Recent findings suggest that clinical cancer progression driven by CSCs may contribute to the failure of existing therapies to consistently eradicate malignant tumors. If the CSCs hypothesis is viable, many aggressive behaviors of cancer cells, such as chemoresistance, like MDR and metastasis, may be better understood. The subsequent fundamental processes of self-renewal and quiescence, proliferation and differentiation as well as apoptosis are governed by a huge number of signalling proteins and enzymes. Thus, under normal physiologic conditions, cellular homeostasis is skilfully sustained by a delicate balance between processes such as self-renewal, proliferation and differentiation versus apoptosis or cell-cycle arrest in progenitor stem cells. In CSCs, this balance is significantly perturbed; hence, the growth and progression mechanisms as well as velocity are constitutively activated whereas the self-defence system, including apoptosis, is sharply attenuated.

Current CSCs research is focusing on the identification of CSCs in solid tumors, since stem cells in hematopoietic malignancies such as leukemia have been well characterized [7]. However, many difficulties are encountered when exploring the existence of CSCs in solid tumors, due to the inaccessibility of tumor cells and the lack of appropriate functional assays [8]. An important breakthrough in the study of solid tumor CSCs was the identification of breast cancer CSCs and their biomarkers by Clarke and his colleagues in 2003 [9]. Since then, CSCs have been reported in neoplasms of brain, prostate, lung, colon, pancreas, liver, melanoma, and skin [10]. Among them, the breast CSCs model with well-defined biomarkers is more advanced than in other types of cancers [11]. Using this model, molecular signatures and signaling pathways have been further explored [12].

There are three main characteristics that define CSCs: (1) differentiation, which provides the ability to give rise to a heterogeneous progeny, (2) self-renewal capability that maintains an intact stem cell pool for expansion, and (3) homeostatic control that ensures an appropriate regulation between differentiation and self renewal according to the environmental stimuli and genetic constraints of each organ tissue, which accounts for the tissue specificity of CSCs. Currently, xenograft assays for different organ sites have been established for testing CSCs. As suggested by the AACR Workshop on Cancer Stem Cells in 2006, the orthotopic xenograft assay

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is considered the golden standard for the identification of CSCs [13]. This type of assay allows reliable testing for all three characteristics of CSCs. In current studies, cancer cells from either tumor tissues or cell lines are initially sorted by specific cell surface markers. Usually, cells that express stem cell markers in tumor tissues are designated as stem-like cells (SLC). The selected cell population is then injected into experimental animals for tumorigenesis testing. If as few as 100–500 cells of the selected cell population are tumorigenic, the featured cell surface markers can serve as CSC-specific biomarkers. In a breast cancer study by Al-Hajj et al. [14], human breast cancer tissues or cells with or without expression of CD44 and CD24 were injected into the mammary fat pad of immune-deficient nonobese diabetic/severe combined immune-deficient (NOD/SCID) mice, which have greater immune deficiency than nude mice. Using this model, the breast CSC-specific biomarkers CD44+/CD24− were determined. Similar xenograft assays in NOD/SCID mice were used to identify CSCs of brain, colon, and lung with a CD133+ profile [15]. Not only the NOD/SCID mouse models but also nude mice are choices for an orthotopic xenograft assay. Visvader and Lindeman have recently summarized mouse models and CSC markers used for isolation of CSC, including CD133, CD44, ALDH1A1, and epithelial cell adhesion molecule (EpCAM) [16]. As shown in Table 1, there is no universal CSC marker for all types of cancer. CSC markers may be tumor type specific, depending on the niche of each type of CSC. In addition to in vivo assays for CSC identification, many *in vitro* experiments have also provided evidence for the existence of CSCs. For example, studies by Collins et al. focused on a cell population in patients' tumor tissues featuring CD44+/integrinα2β1high/CD133+ [17]. These cells were examined by colony-formation and long-term serial culture assays and showed self renewal and regeneration of phenotypically mixed populations. Accumulating evidence suggests that CSCs contribute not only to tumor initiation, but also to aggressive tumor behaviors such as chemoresistance and metastasis.

Table 1. Putative CSC makers in solid tumors.

CSC markers	Tumor types	% CSC markers in tumor cells	Minimal cell no. for tumor formation
CD44+/CD24−/low	Breast	11–35	200
CD44+	Head and neck	0.1–42	5000
	Prostate	0.3–38	100
CD44+/EpCAMhi	Colon	0.03–38	200
CD44+/CD24−/ESA+	Pancreas	0.2–0.8	100
ALDH1+	Breast	3−10	500
CD133+	Brain	6−29	100
	Brain	2-3	500
	Colon	1.8−25	200
	Colon	0.7−6	3000
	Head and neck	0.8−4.2	1000
	Pancreas	1−3	500
	Lung	0.32−22	10^4
Side population	Prostate	0.05−0.2	100
ABCG5+	Melanoma	1.6−20	10^6

Targeted therapies against CSC are currently being developed. However, there is a major difficulty in determining how to effectively attack such a small population within a tumor. A recent study demonstrated that the proportion of tumor cells expressing CD44 (*see above*) increased in human breast cancers 12 weeks after chemotherapy, and these cells had a great propensity for self-re-newal as measured by their ability to form mammospheres in culture and tumors in mice [18]. An increase of CSC was also observed in xenograft models of human colorectal cancer after chemotherapy [19] and in human gliomas after radiation [20]. Thus, time-dependent changes in CSC may have important implications for therapeutic response.

1.1. CSC-like cells as a chemoresistant population

It has been noted that although chemotherapy kills the majority of cancer cells in tumor tissues, it may leave a population of cells behind. These cells overexpress the ATP-binding casstte (ABC) drug transporters which protect cancer cells from damage by cytotoxic agents. Coincidently, a side population (SP) of tumor cells which are defined by their inability to accumulate the fluorescent dye Hoechst 33342 due to overexpression of the ABC transporter ABCG2 has been confirmed to hold CSC features in several types of cancers including hematopoietic, prostate, and glioma CSCs [21]. ABCG2 and other ABC transporter proteins, therefore, have served as CSC markers [22] (see Table 1). Chemoresistant activity has been identified in some CSC-like cell populations. For example, a study of a colorectal cancer cell line that is resistant to 5-fluorouracil (5FU) and oxaliplatin by Dallas et al. showed 5- to 22-fold enrichment of a double CSC marker CD133+/CD44+ population [23]. Another study by Hermann et al. showed that human pancreatic cells that survived prolonged treatment with gemcitabine had a 50-fold increase in a CD133+ population [24].

Considering CSCs a target population for the treatment of human cancer has opened new directions for research efforts in the field. The development of inhibitors against the ABC transporter ABCG2 has been explored in clinical studies [25]. On the other hand, targeting specifically activated signaling pathways in CSCs may provide an effective strategy to eliminate this cell population. Dallas et al. reported that chemoresistant colorectal cancer CSC-like cells showed increased expression of insulin-like growth factor-1 receptor (IGF-1R). This cell population responded to inhibition by an IGF-1R monoclonal antibody more effectively than its nonresistant counterpart [26]. Several signaling pathways, including the Wnt, TGF-β, and CXCR4 pathways, have been suggested to be activated in CSCs [27]. Therapeutically targeting these pathways deserves further investigation.

### 1.2. Migrating or metastatic cancer stem cells

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The existence of metastatic cancer stem cells (mCSCs) was first hypothesized in 2005 by Brabletz et al., based on their observations in colorectal cancer [28]. They proposed that there are two forms of CSCs in tumor progression - stationary CSC (sCSC) and mobile or migrating CSC (mCSC). They proposed that sCSCs are embedded in epithelial tissues or epithelial-based tumors and cannot disseminate. In contrast, mCSCs, which are derived from sCSC by acquiring a transient epithelial-mesenchymal transition (EMT), are located at the tumor-host interface and mediate tumor cell metastasis. In a colorectal cancer model, Brabletz et al. observed that not only the expression levels of EMT-related biomarkers but also their locations in the tumor nest were significantly associated with metastasis. They found that loss of E-cadherin (E-cad) usually resulted in nuclear localization of β-catenin, which is a typical feature of EMT, and nuclear β-catenin was accumulated in dedifferentiated tumor cells at the tumor-host interface. The authors then interpreted these observations in the context of the sCSC and mCSC hypotheses, suggesting that sCSC and mCSC are responsible for formation of the primary tumor and metastasis, respectively. Both sCSC and mCSC can lead to differentiation and tumor heterogeneity. Particularly, metastatic tumors generated from mCSC may experience a mesenchymal-epithelial transition (MET) in the metastatic organ site, which may explain why EMT can not be clearly observed pathologically in many metastatic lesions. In fact, the mCSC hypotheses can be used to explain the "heterogeneous morphology of the primary tumor and how metastases can recapitulate the heterogeneity in differentiation" and "tumor-cell dormancy and disease recurrence" [29]. Two recent publications support the mCSC hypotheses. Mani et al. reported that the stem-like cells identified in breast cancer were associated with EMT markers [30]. A CD133+/CXCR4+ stem-like population isolated by Hermann et al. was suggested to be essential for metastasis of pancreatic cancer [31].

1.3. Hierarchical and stochastic models of CSCs in solid tumors

Although the concept of developmental hierarchy of solid tumors has been discussed in several papers, the hypothetical hierarchical model of CSC/progenitors was clearly proposed in 2007 by Tang et al. based on their studies in prostate CSCs [32]. This model described a hierarchical organization of phenotypically and functionally distinct cells at different stages of prostate tumor maturation. Their study demonstrated that a highly purified CD44+ population was still heterogeneous and enriched in tumorigenic and metastatic progenitors. That is, not only CSC but also progenitors can be tumorigenic in the NOD/SCID mouse model. These two types of tumor cells share the common marker CD44+, but they can be distinguished by other well-defined markers including ABCG2+ and α2β1+, which are specific for tumor progenitors. Recently, Odoux et al. identified chromosomal instability that usually supports a stochastic

model in the mCSC population isolated from liver metastasis of colon cancer [33]. They, therefore, proposed a new model which suggested that both stochastic and hierarchical models can be used to explain the mCSC population (Figure 1).
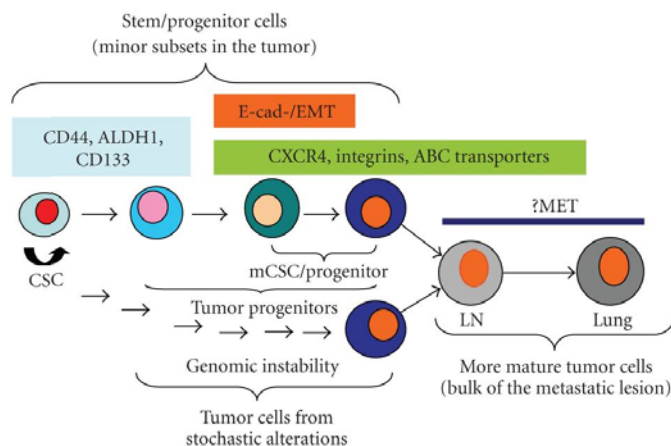
Figure 1. Hierarchical and stochastic models of CSC in progression of solid tumors.
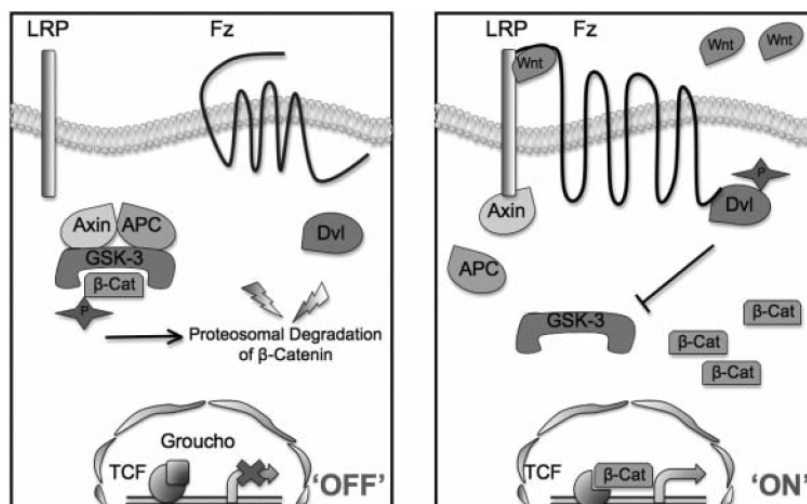
In addition to intrinsic pathways regulating stem cell functions, normal and malignant stem cells are regulated by extrinsic signals generated in the microenvironment or CSC niche. In several cases, this niche is composed of immune cells, mesenchymal elements that include fibroblasts, endothelial cells, adipocytes, and extracellular matrix components [34]. These components play an important role in normal organ development and carcinogenesis. If the cellular microenvironment plays an important role in the regulation of CSC growth and survival, then strategies aimed at interfering with these interactions represent a rational approach to target CSCs.

2. Current strategies to prevent CSCs growth and progression

The CSCs concept has important implications for understanding carcinogenesis as well as for the development of cancer therapeutics. As mentioned above, according to this concept, tumors are initiated and maintained by a cellular subcomponent that displays stem cell properties. These properties include self-renewal, which drives tumorigenesis, and differentiation (albeit aberrant), which contributes to tumor cellular heterogeneity. The existence of CSCs has been described in a variety of hematologic and solid tumors including those of the breast, brain, colon, pancreas, lung, liver, and head and neck [35]. In addition to driving tumorigenesis, CSCs may contribute to tumor metastasis as well as to tumor recurrence after treatment [36]. Several recent studies have questioned the rarity of tumor cells with stem cell properties and tumor-initiating capacity as well as assays used to access these cell populations [37]. Nevertheless, *in vitro* and animal models have demonstrated that breast CSCs are relatively resistant to both radiation and chemotherapy [38]. This preclinical evidence has been supported by clinical studies

demonstrating that the percentage of breast CSCs increased after neoadjuvant chemotherapy [39]. Furthermore, the resistance of chronic myelogenous leukemia stem cells to imatinib (Gleevec), a BCR-ABL inhibitor, indicates that CSCs may also be resistant to some molecularly targeted agents. These studies suggest that the development of more effective cancer therapies may require effective targeting of the CSC population. One of the therapeutic strategies being pursued to target CSCs involves inhibition of self-renewal or survival pathways in these cells, for example Wnt/β-catenin, Hedgehog and Notch pathways. Such strategies may be limited by the role of these pathways in normal stem cell function, which could result in systemic toxicities from pathway inhibition.

Intracellular signalling mediated by secreted Wingless and Int1 (Wnt) proteins is essential for the establishment of cell fates and proper tissue patterning during embryo development and for the regulation of tissue homeostasis in adult tissues. In addition, Wnt signaling is an essential cellular communication pathway that regulates proliferation and differentiation of non-neoplastic stem/progenitor cells in various tissues including retina, skin, and gut [40]. However, it has also been shown that this signaling route is deeply implicated in cancer as well as in CSCs self-renewal and differentiation [41]. Aberrant activation of Wnt signalling pathways has been directly linked to the genesis of different tumours. Wnt ligands are secreted glycoproteins that bind to the co-receptors frizzled (FZD) and low density lipoprotein receptor-related protein 5 and 6 (LRP5/6). In the "canonical" Wnt pathway (Fig. 2), secreted Wnt ligands bind to their receptors, which activates Disheveled (Dvl) and initiates a series of molecular events that lead to increased β-catenin [42]. Nuclear translocation of β-catenin allows it to bind to T-cell factor/lymphoid enhancer binding factor (TCF/LEF) transcription factors, which activates transcription of Wnt target genes, including genes that control cell division, apoptosis, stem cell phenotype, and metastasis [43]. In the absence of Wnt ligands, β-catenin levels are maintained at low levels through phosphorylation by the adenomatous polyposis coli (APC)-axin-glycogen synthase kinase 3 beta (GSK3β) protein complex. In many tumors, mutational activation of APC and axin, or constitutive activation of β-catenin, leads to elevated Wnt signaling. For example, attention has been drawn to control of tumor-initiating colorectal cancer (CRC) stem cell self-renewal, proliferation, and differentiation by the Wnt and transforming growth factor (TGF)-beta pathways [44]. Disruption of Wnt signaling, via loss of APC, is among the earliest events in the multistage progression of CRC and likely occurs in basal crypt stem cells, generating a neoplastic cell population that then expands upward to occupy the rest of the crypt.

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Figure 2. The canonical Wnt transduction pathway (Proteosomal degradation of β-catenin via its phosphorylation occurs in the absence of Wnt ligands. Downstream, Wnt target genes are maintained repressed ('OFF'). Degradation of active β-catenin is reduced upon the binding of Wnt`s. Accumulation and translocation of β-cateuiu into the nucleus lead to binding to T-cell factors and activation of target genes ('ON')).

In addition, human colon cancers often start as benign adenomas through loss of APC, leading to enhanced β-catenin (β-Cat)/Tcf function. In particular, it was found that epithelial cells of human colon carcinomas (CCs) and their stem cells of all stages harbour an active Hedgehog-Gli (Hh-Gli) pathway [45]. Thus, it was clearly indicated that this signaling route plays a key and essential role in promoting CC growth, stem cell self-renewal and metastatic behavior in advanced cancers. It has also been found that the Wnt/β-catenin signalling pathway is also implicated in the development of leukemia stem cells in AML [46].

Casein kinase 1 delta and epsilon (CK1δ/ε) are key regulators of diverse cellular growth and survival processes including Wnt signaling, DNA repair and circadian rhythms [47]. Recent studies suggest that they have an important role in oncogenesis. RNA interference screens identified CK1ε as a pro-survival factor in cancer cells *in vitro* and the CK1δ/ε-specific inhibitor IC261 is remarkably effective at selective, synthetic lethal killing of cancer cells. The recent development of the nanomolar CK1δ/ε-selective inhibitor, PF670462 (PF670) and the CK1ε-selective inhibitor PF4800567 (PF480) offers an opportunity to further test the role of CK1δ/ε in cancer (Fig. 3).

PF-670462   **PF-4800567**   **IC-261**
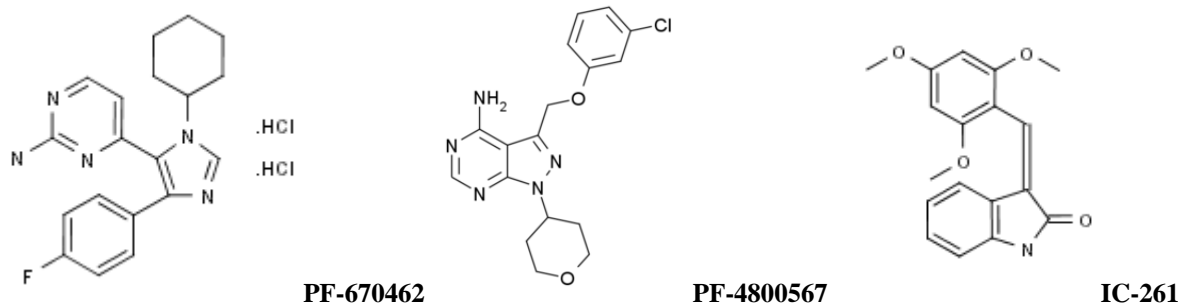
Fig. 3. Small molecule CK1δ/ε inhibitors

Unexpectedly, and unlike IC261, PF670 and PF480 were unable to induce cancer cell death. PF670 is a potent inhibitor of CK1δ/ε in cells; nanomolar concentrations are sufficient to inhibit CK1δ/ε activity as measured by repression of intramolecular autophosphorylation, phosphorylation of disheveled2 proteins and Wnt/β-catenin signaling. Likewise, small interfering RNA knockdown of CK1δ and CK1ε reduced Wnt/β-catenin signaling without affecting cell viability, further suggesting that CK1δ/ε inhibition may not be relevant to the IC261-induced cell death. Thus, while PF670 is a potent inhibitor of Wnt signaling, it only modestly inhibits cell proliferation. In contrast, while sub-micromolar concentrations of IC261 neither inhibited CK1δ/ε kinase activity nor blocked Wnt/β-catenin signaling in cancer cells, it caused a rapid induction of prometaphase arrest and subsequent apoptosis in multiple cancer cell lines. In a stepwise transformation model, IC261-induced killing required both overactive Ras and inactive p53. IC261 binds to tubulin with an affinity similar to colchicine and is a potent inhibitor of microtubule polymerization. This activity accounts for many of the diverse biological effects of IC261 and, most importantly, for its selective cancer cell killing. Three crystal structures of the kinase domain of human CK1δ, one apo and two complexed with a potent and selective CK1δ/ε inhibitor PF670462 in two different crystal forms were also reported (Fig. 4). These structures provide a molecular basis for the strong and specific inhibitor interactions and suggest clues for further development of CK1δ/ε inhibitors [48].
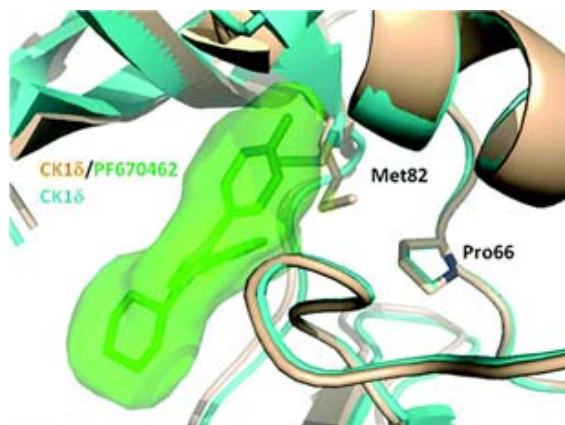
Fig. 4. Crystal structure of *h*CK1δ in complex with PF670462 (3UZP)

The chemotherapeutic agents that inhibit activation of Gli transcription factors have emerged as promising novel therapeutic drugs for pancreatic cancer. GDC-0449 (Vismodegib, Fig. 5), orally administrable molecule belonging to the 2-arylpyridine class, inhibits SHH signaling pathway by blocking the activities of Smoothened. This molecule was tested *in vitro* and the molecular mechanisms by which GDC-0449 regulates human pancreatic CSC characteristics were examined [49].
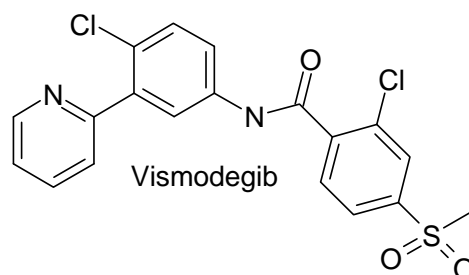
Fig. 5. the structure of GDC-0449

It was found that GDC-0499 inhibited cell viability and induced apoptosis in three pancreatic cancer cell lines and pancreatic CSCs. This inhibitor also suppressed cell viability, Gli-DNA binding and transcriptional activities, and induced apoptosis through caspase-3 activation and PARP cleavage in pancreatic CSCs. GDC-0449-induced apoptosis in CSCs showed increased Fas expression and decreased expression of PDGFRα. Furthermore, Bcl-2 was down-regulated whereas TRAIL-R1/DR4 and TRAIL-R2/DR5 expression was increased following the treatment of CSCs with GDC-0449. Suppression of both Gli1 plus Gli2 by shRNA mimicked the changes in cell viability, spheroid formation, apoptosis and gene expression observed in GDC-0449-treated pancreatic CSCs. Thus, activated Gli genes repress DRs and Fas expressions, up-regulate the expressions of Bcl-2 and PDGFRα and facilitate cell survival. The obtained data suggest that GDC-0499 can be used for the management of pancreatic cancer by targeting pancreatic CSCs.
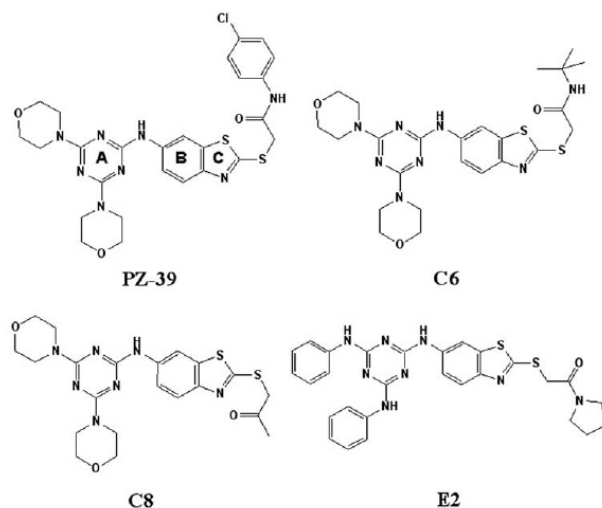
Recent data have implicated DAX-1, an unusual orphan nuclear receptor/transcription factor encoded by NR0B1 gene, in embryonic stem cell and cancer biology [50]. The role of DAX-1 in the regulation of development and function of the adrenal cortex, reproductive axis, embryonic stem cells and a few types of cancer has been described therein. The unusual structure and restricted expression pattern of DAX-1 may offer unique opportunities for drug discovery particularly in the field of CSCs.

It is well known that Notch receptor signaling pathways play an important role not only in normal breast development but also in breast cancer development and progression. The role of

Notch receptors in stem cell activity in breast cancer cell lines and nine primary human tumor samples has recently been revealed in [51]. It was found that Notch4 signaling activity was 8-fold higher in stem cell-enriched cell populations compared with differentiated cells, whereas Notch1 signaling activity was 4-fold lower in the stem cell-enriched cell populations. Pharmacologic or genetic inhibition of Notch1 or Notch4 reduced CSCs activity *in vitro* and reduced tumor formation *in vivo*, but Notch4 inhibition produced a more robust effect with a complete inhibition of tumor initiation observed. These findings suggest that Notch4-targeted therapies will be more effective than targeting Notch1 in suppressing breast cancer recurrence, as it is initiated by breast cancer stem cells.

The Ras/Raf/MEK/ERK pathway is often activated by genetic alterations in upstream signaling molecules. Integral components of this pathway such as Ras and B-Raf are also activated by mutation. The pathway has profound effects on proliferative, apoptotic and differentiation pathways; this route can often be effectively silenced by MEK inhibitors. Recently, in addition to the prevention of cellular aging, the role of these inhibitors in the development of CSCs has also been suggested [52].

Among the biological targets which have been found to play a role in CSC growth and development, the ABC protein family, including ABCB1, ABCC1 and ABCG2 subtypes, are of the most prominent. The recent addition, - human ABCG2, a member of the ATP-binding cassette transporter superfamily, has been suggested to be deeply implicated in protecting CSCs, resulting in drug resistance, including MDR (a major problem in successful treatment of cancers), and failure of cancer chemotherapy [53]. It is very valuable that knockout of ABCG2 had no apparent adverse effect on the mice. Thus, ABCG2 is an ideal target for development of chemo-sensitizing agents for better treatment of drug resistant cancers and helping eradicate cancer stem cells. In particular, it was found that unlike any previously known ABCG2 transporter inhibitors, PZ-39 and its structural analogues (Fig. 6) have a novel two-mode action by inhibiting ABCG2 activity, an acute effect, and by accelerating lysosome-dependent degradation, a chronic effect. PZ-39 is potentially a valuable probe for structure-function studies of ABCG2 and a lead compound for developing therapeutics targeting ABCG2-mediated MDR in combinational cancer chemotherapy.

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Figure 6. Schematic 2-dimensional chemical structures of PZ-39 and related compounds.

Telomerase activity is essential for the continued growth and survival of malignant cells, therefore inhibition of this activity presents an attractive target for anti-cancer therapy. The telomerase inhibitor GRN163L, was shown to inhibit the growth of cancer cells both *in vitro* and *in vivo*. It was suggested that telomerase inhibitors can be regarded as promising therapeutics against CSCs, without harmful effects toward particularly mesenchymal stem cells (MSCs) [54].

Evidence [55] suggests that several solid tumors, including breast cancer, possess a rare population of CSCs capable of extensive self-renewal that contribute to metastasis and treatment resistance. It has recently been reported that the development of a strategy to target CSCs through the blockade of the IL-8 receptor CXCR1 may lead to benefit therapeutic outcomes For example, in two human breast cancer cell lines, CXCR1 blockade using either a CXCR1-specific blocking antibody or repertaxin, a small-molecule CXCR1 inhibitor, selectively depleted the CSC population. Furthermore, this was followed by the induction of massive apoptosis in the bulk tumor population via FASL/FAS signaling. The effects of CXCR1 blockade on CSC viability and on FASL production were mediated by the FAK/AKT/FOXO3A pathway. In addition, repertaxin was able to specifically target the CSC population in human breast cancer xenografts, retarding tumor growth and reducing metastasis. This data therefore suggest that CXCR1 blockade may provide a novel means of targeting and eliminating breast CSCs.

Deregulated expression/activation of transcription factors is a key event in the establishment and progression of human cancer. Furthermore, most oncogenic signaling pathways converge on sets of transcription factors that ultimately control gene expression patterns resulting in cancer development, progression, and metastasis [56]. Activation targets of Nanog, Oct-4, Sox2 and c-Myc are more frequently overexpressed in certain tumors. In the absence of bona fide cancer stem cell lines, human embryonic stem cells, which have similar properties to cancer and cancer stem cells, have been an excellent model throwing light on the

anticancer affects of various putative anticancer agents. Nanog, Sox2 and Oct-4 are transcription factors which are essential to maintaining the pluripotent embryonic stem cell phenotype. Oct-4 and Sox2 bind to the Nanog promoter in living mouse and human ESCs. Nanog, Oct-4 and Sox2 co-occupy and regulate their own promoters together with other developmental genes with diverse functions and collaborate to form an extensive regulatory circuitry including autoregulatory and feed-forward loops. A high level of Nanog is a key regulator of ESC self-renewal and puripotency. Nanog-deficient ES cells and embryos lose their pluripotency. Nanog overexpression leads to the clonal expansion of ES cells through circumvention of the LIF-dependent Stat-3 pathway and sustained Oct-4 expression levels. Genome-wide gene expression profiling shows that Nanog is expressed at high levels in testicular carcinoma in situ and germ cell tumors. Positive correlations of Oct-4, Nanog, or CD133 expression on tumor stage were shown on oral squamous cell carcinoma patient tissues. Recently were demonstrated that inhibition of Nanog by shRNA enhanced the inhibitory effects of EGCG and SFN on self-renewal capacity of CSCs, suggesting its requirement for self-renewal of CSCs. Medulloblastomaassociated CSCs selected by serum-free medium with bFGF and EGF can form 3D spheroids and display enhanced self-renewal and highly co-expressed stem cell genes (Oct4, Nanog, Nestin, and Musashi-1) as well as anti-apoptotic genes (Bcl-2 and Bcl-XL). These finding suggest that inhibition of pluripotency maintaining transcription factor such as Nanog could be a novel strategy to kill CSCs. [57]

The c-MYC - proto-oncogene encodes a transcription factor, which regulates the xpression of cellular targets involved in a wide range of diverse cellular functions, including cell growth, proliferation, loss of cell-cell contact, loss of differentiation and angiogenesis [58,59]. The recent studies have shown that altered expression of several stem cell related factors may play different roles in renal cell carcinoma (RCC). C-MYC may function as an oncogene and OCT4, KLF4, NANOG and SOX2 as tumor suppressors [60].

Ubiquitin ligases, critical to virtually all cellular signaling systems, alter the degradation or trafficking of most proteins in the cell, and indeed broad perturbation of this system, through inhibition of the proteosome, is a successful cancer treatment. The authors [61] examined several glioblastoma stem cell isolates pre- and postdifferentiation to elucidate the phenotypic effects following shRNA knockdown of ubiquitin ligases. The results were analyzed using high-content imaging (HCI) and identified ubiquitin ligases capable of inducing both CSC differentiation and apoptosis. Quite often these effects were specific to CSCs, as ubiquitin ligase knockdown in terminally differentiated progeny yielded markedly different results. The resolution of HCI at the subpopulation level makes it an excellent tool for the analysis of CSC phenotypic changes induced by shRNA knockdown and may suggest additional methods to target these cells for

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death or differentiation. Various roles of ubiquitin in Wnt pathway regulation were described by Tauriello DV and Maurice MM in [62]. Many challenges lie ahead to address the mechanistic details (attachment sites, chain linkages) by which ubiquitin controls protein function in Wnt signaling and how E3 ligase and DUB activity is controlled in time and space. Among these, the identification of ubiquitin-binding proteins that recognize conjugated ubiquitin moieties and control assembly of complex signaling networks will be of critical importance. Convincing evidences have shown that Itch E3 ubiquitin ligase is a bona fide binding partner and negative regulator of LATS1 [63]. The large tumor suppressor 1 (LATS1) is a serine/threonine kinase originally identified as a homolog of Drosophila tumor-suppressor LATS and tumor suppressor found down-regulated in a broad spectrum of human cancers. LATS1 is a central player of the emerging Hippo-LATS suppressor pathway, which plays important roles in cell proliferation, apoptosis, and stem cell differentiation. Hippo-LATS pathway is also implicated in a number of fundamental biological processes, such as animal organ size control, color recognition in the eye, cell-cell competition, and neural dendrite formation and maintenance. It has been found that inactivation of LATS1 caused cytokinesis defects, genetic instability, and polyploidy, all of which are hallmarks of cancer. Moreover, reduced expression and promoter methylation of LATS1 are also found in various cancers, including leukemia, breast cancer, astrocytoma, and soft tissue sarcoma. In addition, it was identified that Itch, a HECT class E3 ubiquitin ligase, acts as a unique binding partner of LATS1. Itch forms the complex with LATS1 both *in vitro* and *in vivo* through the PPxY motifs of LATS1 and the WW domains of Itch. Significantly, it was found that overexpression of Itch promoted LATS1 degradation by polyubiquitination through the 26S proteasome pathway. On the other hand, knockdown of endogenous Itch by shRNAs provoked stabilization of endogenous LATS1 proteins. Finally, through several functional assays, it was revealed that change of Itch abundance alone is sufficient for altering LATS1-mediated downstream signaling, negative regulation of cell proliferation, and induction of apoptosis. Consequently, it has been suggested that Itch may play a role in defining the fine balance between cell death and cell survival in human cancers. Hence, development of strategies that specifically disrupt LATS1 and Itch interaction may be useful for driving tumor suppression in cancers.

Histone modification determines epigenetic patterns of gene expression with methylation of histone H3 at lysine 4 (H3K4) often associated with active promoters. LSD1 is a lysine-specific demethylase 1; also known as KDM1A and AOF2 that suppresses gene expression by converting di-methylated H3K4 to mono- and un-methylated H3K4. LSD1 occupies the promoters of a subset of developmental genes that contain bivalent domains and are co-occupiedby OCT4 and NANOG in human embryonic stem cells, where it controls the levels of

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H3K4 methylation through its demethylase activity. Thus, LSD1 has a role in maintaining the silencing of several developmental genes in human embryonic stem cells by regulating the critical balance between H3K4 and H3K27 methylation at their regulatory regions [64]. Thus, specific bioactive small inhibitors of LSD1 that enhance H3K4 methylation and derepress epigenetically suppressed genes *in vivo* have been developed [65]. Strikingly, these compounds inhibited the proliferation of pluripotent cancer cells including teratocarcinoma, embryonic carcinoma, and seminoma or embryonic stem cells that express the stem cell markers Oct4 and Sox2, while displaying minimum growth inhibitory effects on non-pluripotent cancer or normal somatic cells. RNAi-mediated knockdown of LSD1 expression phenocopied these effects, confirming the specificity of small molecules and further establishing the high degree of sensitivity and selectivity of pluripotent cancer cells to LSD1 ablation. In support of these results, the authors found that LSD1 protein level is highly elevated in pluripotent cancer cells and in human testicular seminoma tissues that express Oct4. Using these novel chemical inhibitors as probes, the authors established LSD1 and histone H3K4 methylation as essential cancer-selective epigenetic targets in pluripotent cancer cells that have stem cell properties.

Other data indicated that LSD1 and HDACs cooperated to regulate key pathways of cell death in Glioblastoma multiforme (GBM) cell lines but not in normal counterparts, and they validate the combined use of LSD1 and HDAC inhibitors as a therapeutic approach for GBM [66,67]. In particular, it was suggested that that LSD1 intimately interacts with histone deacetylases (HDAC) in human breast cancer cells [68]. Inhibition of histone demethylation and deacetylation exhibits cooperation and synergy in regulating gene expression and growth inhibition, and may represent a promising and novel approach for epigenetic therapy of breast cancer [69].

Selective inhibitors of Jumonji domain-containing protein (JMJD) histone demethylases are candidate anticancer agents as well as potential tools for elucidating the biological functions of JMJDs. On the basis of the crystal structure of JMJD2A and a homology model of JMJD2C (Fig. 7), were designed and prepared a series of hydroxamate analogues bearing a tertiary amine. Enzyme assays using JMJD2C, JMJD2A, and prolyl hydroxylases revealed that several hydroxamate analogs are potent and selective JMJD2 inhibitors, showing 500-fold greater JMJD2C-inhibitory activity and more than 9100-fold greater JMJD2C-selectivity compared with the lead compound N-oxalylglycine (**2**) (Fig. 8).
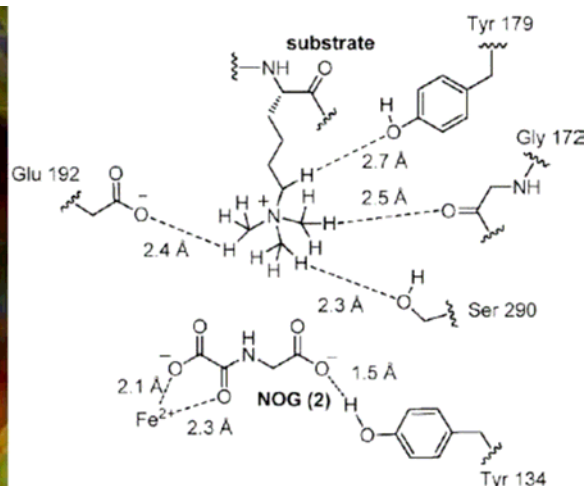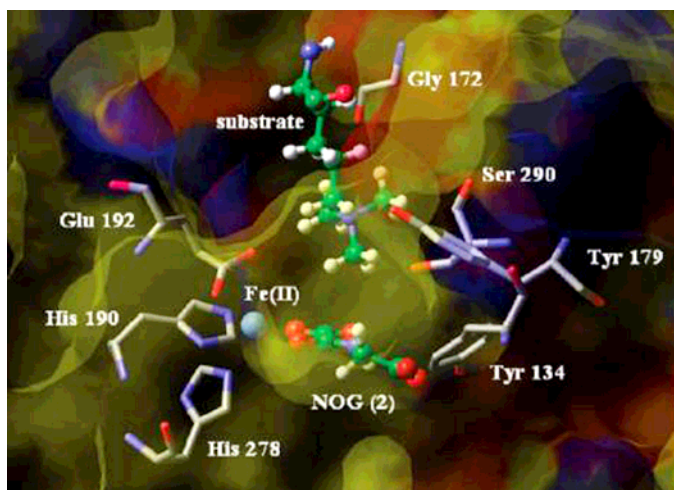
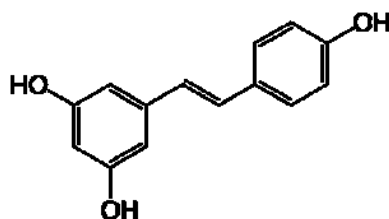**Figure 7.** View of the active site of the JMJD2C homology model.

Compounds **1** and **2**, prodrugs of compound **3**, each showed synergistic growth inhibition of cancer cells in combination with an inhibitor of lysine-specific demethylase 1 (LSD1). These findings suggest that combination treatment with JMJD2 inhibitors and LSD1 inhibitors may represent a novel strategy for anticancer chemotherapy [70].

Compounds 1- R = Me
Compounds 2 - R = Ac
Compounds 3 - R = H
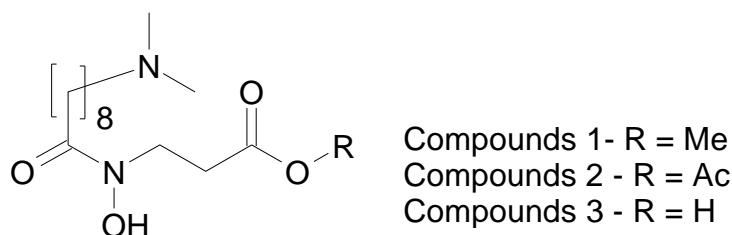
Figure 8. Examples of hydroxamate analogs with an inhibition activity against JMJD2 in a CSC model

Asymmetric cyclopropanation of styrenes by tert-butyl diazoacetate followed by ester hydrolysis and Curtius rearrangement gave a series of tranylcypromine analogues as single enantiomers. The o,- m- and p-bromo analogues were all more active than tranylcypromine in a LSD1 enzyme assay. The m- and p-bromo analogues were micromolar growth inhibitors of the LNCaP prostate cancer cell line as were the corresponding biphenyl analogues prepared from the bromide by Suzuki crosscoupling [71].

Resveratrol (3,4',5-trihydroxy-trans-stilbene; RV) (Fig. 9), an ingredient of wine, exhibits a broad spectrum of antiproliferative effects against human cancer cells.

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Figure 9. 3,4',5-trihydroxy-trans-stilbene; RV

Molecular mechanisms by which resveratrol inhibits stem cell characteristics of pancreatic CSCs derived from human primary tumors and Kras(G12D) transgenic mice have been studied in [72]. The effects of resveratrol towards the expression of Nanog, Sox-2, cMyc and Oct-4 in human pancreatic CSCs expressing CD44+/CD24+/ESA+ were particullary investigated. Cancer stem cells have been shown to exhibit drug resistance properties by expressing multidrug resistance genes such as ABCG2. The obtained data suggest that inhibition of ABCG2 by resveratrol could be beneficial in enhancing the biological effects of resveratrol alone or in combination with other drugs. In order to enhance these effects, the core structure was modified by introducing additional methoxyl and hydroxyl groups (Fig. 10).

R1-5=H, OH, OMe
R6=OH, OMe
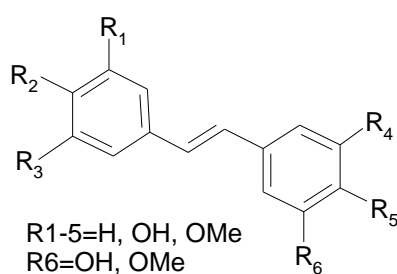
Figure 10. Direct RV analogs

The resulting novel RV analogs, 3,4',5-trimethoxy-trans-stilbene, 3,3',4,5'-tetramethoxy-trans-stilbene and 3,3',4,4',5,5'-hexahydroxy-trans-stilbene were investigated in HT29 human colon cancer cells [73]. Due to these promising results, the investigated RV analogs deserve further preclinical and *in vivo* testing. The results of [74] study indicated that resveratrol is capable of inducing apoptosis in the cancer stem-like cells through suppression of lipogenesis by modulating FAS expression, which highlights a novel mechanism of anti-tumor effect of resveratrol.

Salinomycin is a novel identified cancer stem cells (CSCs) killer. The anticancer activity of salinomycin has evoked excitement due to its recent identification as a selective inhibitor of breast cancer stem cells (CSCs) and its ability to reduce tumor growth and metastasis *in vivo*. In prostate cancer, similar to other cancer types, CSCs and/or progenitor cancer cells are believed to drive tumor recurrence and tumor growth. Thus salinomycin can potentially interfere with the end-stage progression of hormone-indifferent and chemotherapy-resistant prostate cancer. The result shows that salinomycin might be selective therapy for CSCs fraction [75, 76, 77, 78].

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Recent progress in the study of CSCs in solid tumors has provided researchers and clinicians in cancer new concepts to better understand the heterogeneity of this disease with. The following section provides our *in silico* approach to design of novel promising CSCs inhibitors under the cover concept of target diversity.

3. Concept and Applications

CSCs-targeted library design at CDL involves:

• *A combined profiling methodology that provides a consensus score and decision based on various advanced in silico tools covering by the top concept of targeted diversity*:

The following strategy has been applied to design our "First-Step" targeted set:

1. Targeted diversity concept;

2. 2D-similarity approach (Tanimoto metric) to the reported inhibitors of CSCs growth and progression;

3. Unique isosteric and bioisosteric morphing and funneling procedures in designing novel potential CSCs inhibitors with high IP value. We apply CDL's proprietary Chemosoft™ software and commercially available solutions from Accelrys, ChemoSoft, MolSoft, SmartMining, MOE, Daylight and other platforms;

4. Compounds selection based on key structural motifs revealed for reported CSCs agents;

5. Non-trivial peptidomimetics, including α-helix and β-turn mimetics, which are targeted specifically against PPI within the CSCs interface, for example in Wnt signalling route;

6. A particular focus was on *Spiro-*, *sp3*-rich and "*beyond-flatland*" compounds;

7. Topological analogues, e.g. GDC-0449 and Sulindac skeleton mimetics with crutial binding points;

8. A subset of high diversity was included;

9. Analogues of natural polyphenols;

10. ChemDiv's medchem filters

Advanced approaches which are planned to be applied in further design include:

1. Neural Network tools for target-library profiling, in particular Self-organizing Kohonen and Sammon maps, performed in SmartMining Software, Support vector machine (SVM) methodology, etc.;

2. 3D-molecular docking approach;

3. 3D-pharmacophore modeling/searching;

4. Computational-based `in silico` ADME/Tox assessment for novel compounds includes prediction of human CYP P450-mediated metabolism and toxicity as well as many

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pharmacokinetic parameters, such as Brain-Blood Barrier (BBB) permeability, Human Intestinal Absorption (HIA), Plasma Protein binding (PPB), Plasma half-life time ($T_{1/2}$), Volume of distribution in human plasma (V_d), etc. The fundamentals for these applications are described in a series of our recent articles on the design of exploratory small molecule chemistry for bioscreening [for related data visit ChemDiv. Inc. online source: www.chemdiv.com].

• _Synthesis, biological evaluation and SAR study for the selected structures_:

1. High-throughput synthesis with multiple parallel library validation. Synthetic protocols, building blocks and chemical strategies are available;

2. Library activity validation via bioscreening; SAR is implemented in the next library generation.

ChemDiv introduces the concept of Targeted Diversity which is intended for the design of high quality libraries of drug-like compounds that have been focused against various biological targets. Targeted diversity signifies the superposition of highly diverse chemical space on the assortment of divergent families or sub-families of targets and unique biomolecules. These targets may be congener or "orthogonal" (non-overlapping) and include:
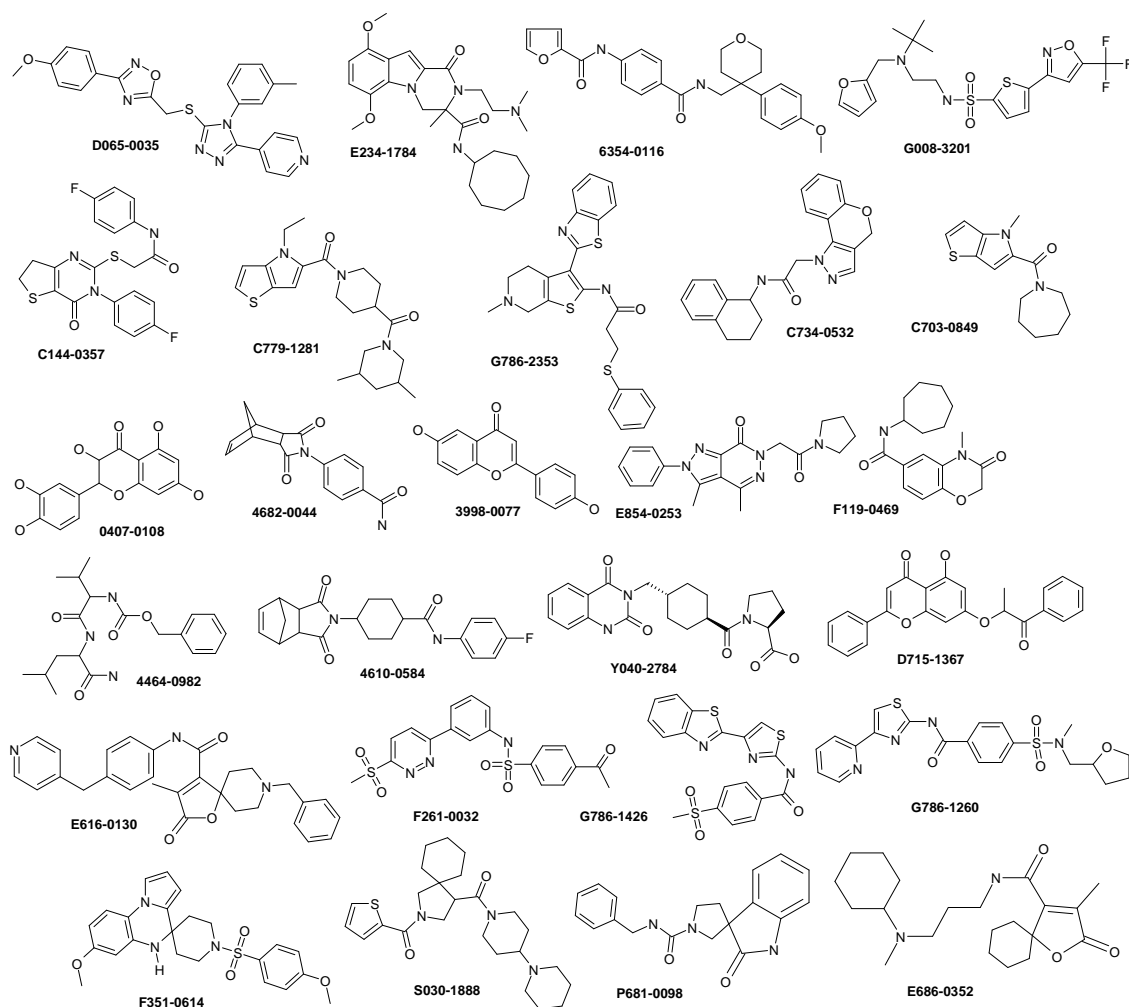
(a) Different classes of targets.

(b) Distinct, structurally unrelated branches of the same target class.

(c) Independent targets.

The different classes of biomolecules are represented by G-protein coupled receptors (GPCR), nuclear hormone receptors (NHR), ligand- and voltage-gated ion channels (LGICh and VGICh), transporters (TR), various enzymes (kinases, proteases, phosphodiesterases, etc.), effector proteins and others. Examples of the branches of related proteins include serine/threonine protein kinases (STPK) and tyrosine kinases (TK) as sub-families of the kinome. An example of independent targets is GPCR-like Smo receptors. The current edition of the Targeted Diversity Library (TDL) is based on approximately 150 small molecule sets. Each of these sets is focused against distinct biological targets belonging to the different classes and sub-families of targets (list of targets selected is shown below) and includes about 5000 individual drug-like molecules. The selection process for these sets involves identifying active ligands/inhibitors as prototypes existing in the patent and research literature or databases and performing bioisosteric replacement strategies, e.g. a known peptide ligand may be substituted with a small non-peptide peptidomimetic. Then a similarity search based on these strategies is conducted within ChemDiv's collection for possible augmentation of the rational set. Other techniques include computer-assisted 3D pharmacophore matching and when possible, _in silico_

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docking experiments. The directed synthesis of new chemotypes with functionality mimicking recognition elements (shapes, "warheads") of known active ligands/inhibitors has also been performed. In some cases, proof of concept has been established with in-house biological data. A special effort has been made to select respective compounds and synthetic templates with good IP potential, as deduced from Beilstein, SciFinder and Markush sub-structure searches. The special rules of ChemDiv's medchem filters (MCF) ensure the high quality and drug-like properties of selected molecules. The first edition of the TDL includes the most diverse compounds (250-750 members) from each of 150 target-specific sets. The current TDL is built around 1,500 diverse chemical templates to yield a library of about 25,000 individual drug-like molecules. Embellishment of the library is an ongoing effort at ChemDiv. Regular updates are being made as newly synthesized compounds become available and pass our QA specifications (>90% purity as established by LC/MS with UV and ELSD). Additionally, new proposals for target-specific sets are being evaluated, tested and made available. Thus, the TDL may provide high-quality hits in screening against "difficult" targets with limited or no structure/ligand information, as well as "eclectic" biological targets, including cellular processes (e.g. apoptosis and cell cycle), signaling pathways (e.g. WNT, Hh, RTK and Ras) or protein-protein interactions (e.g. XIAP, pGPCRs). For the design of our CSC-targeted library we have used a wide spectrum of related biological targets, including receptors and enzymes successfully validated in clinics as well as promising targets which are currently under active investigation in the title field. All these targets are implicated in cancer stem cells growth and progression. The list of core biological targets used for CSC-library design is presented below:

1. Wnt/β-catenin, Hedgehog and Notch pathways;
2. Dvl phosphorylation inhibitors and PPI: Dvl/(Axin,GBP,APC);
3. PPI: Dvl/Frizzled;
4. Rac1 inhibition (Dvl is activated by phosphorylation and poly-ubiquitination, which in turn recruits GSK-3β away from the degradation complex. This allows for stabilization of β-catenin levels, Rac1-dependent nuclear translocation and recruitment to the LEF/TCF DNA-binding factors where it acts as an activator for transcription by displacement of Groucho- HDAC co-repressors.);
5. PPI: Prop1/β-catenin inhibition;
6. LRP5/6 ectodomain antagonists and Axin/Frat-1/LRP5(6) interface (PPI) inhibitors;
7. Secreted Frizzled-related Protein-1 (SFRP-1) mimetics;
8. CK1δ/ε inhibition;
9. CXCR1/4;
10. DAX-1;

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11. MEK;

12. ABCB;

13. ABCC and ABCG;

14. Alox5-related signalling pathway;

15. Hsp90;

16. Omacetaxine and telomerases;

17. Aryl hydrocarbon receptor (AHR), with tranilast and other AHR agonists;

18. Cytokines and inflammatory pathways (e.g., IL-6, IL-8, NF-κB);

19. TGF-β and epithelial-to-mesenchymal transition (EMT) pathways;

20. Growth factors, their receptors and coreceptors (such as neuropilin-1), and signaling components (e.g., tyrosine kinases);

21. Cell-surface markers (CD44 and integrins);

22. NF-κB inhibition;

etc.

Figure 8. Examples of compounds from CSC-targeted library.

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Key statistical indicators calculated for CSC-targeted library are listed below:

- total diversity coeff.: 0.7645;

- number of screens: 5642;

- number of unique heterocycles: 381

A wide range of molecular descriptors was calculated for CSC-targeted library. Several representative examples are presented in Fig. 9.
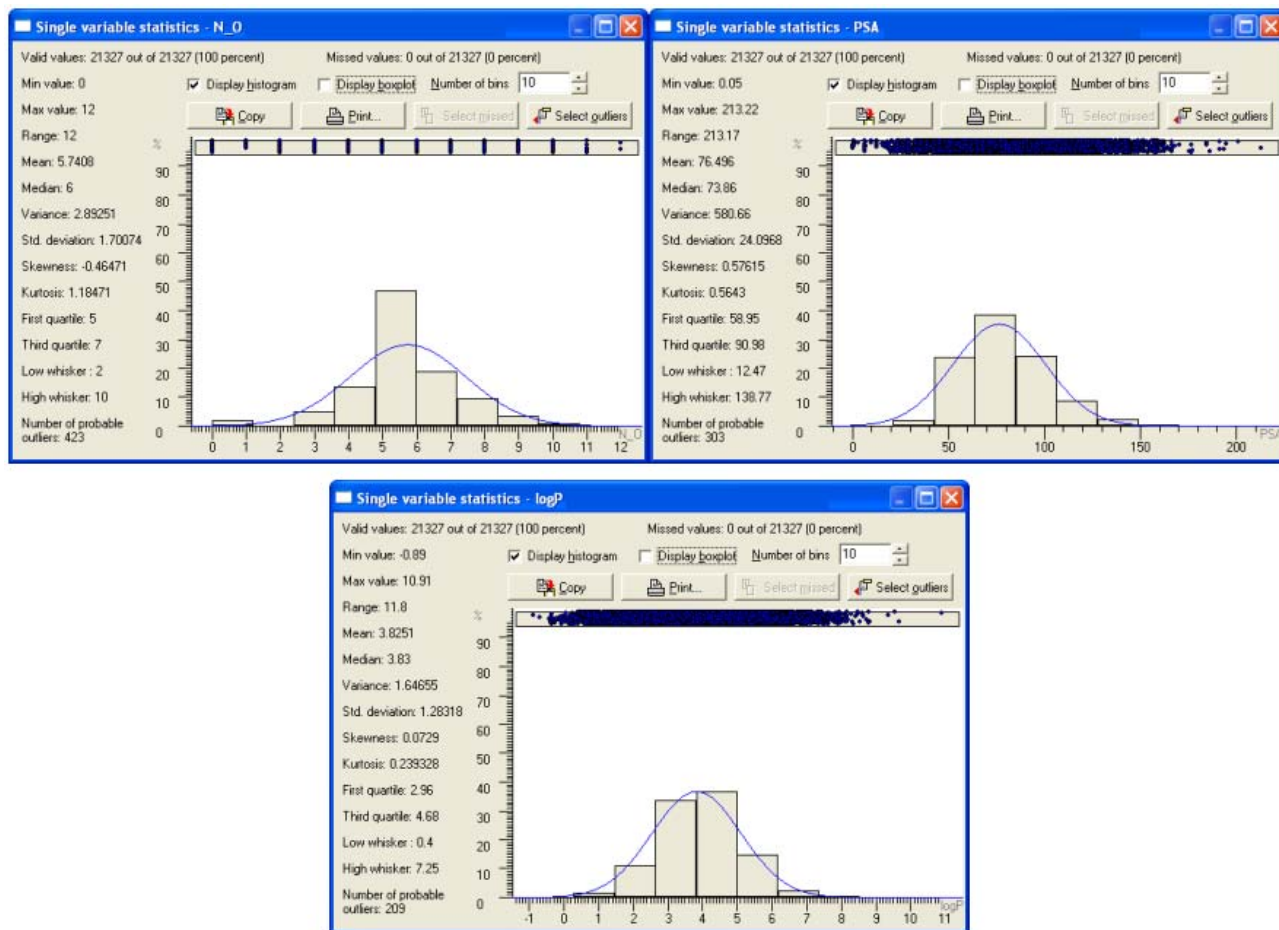

Fig. 9. Distrubution of several descriptors among compounds from CSC-library

Conclusion

Although the concept of CSCs was first proposed more then a century ago, it has attracted a great deal of attention recently due to advances in stem cell biology, leading to the identification of these cells in a wide variety of human cancers. There is accumulating evidence that the resistance of cancer stem cells to many conventional therapies may account for the inability of these therapies to cure most metastatic cancers. The recent identification of stem cell markers and advances in stem cell biology has facilitated research in multiple aspects of cancer stem cell behavior. Stem cell subcomponents have now been identified in a number of human malignancies, including hematologic malignancies and tumors of the breast, prostate, brain, pancreas, head and neck, and colon. Furthermore, pathways that regulate self-renewal and cell

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fate in these systems are beginning to be elucidated. In addition to pathways such as Wnt, Notch and Hedgehog, known to regulate self-renewal of normal stem cells, tumor suppressor genes such as PTEN (phosphatase and tensin homolog on chromosome 10), TP53 (tumor protein p53), DAX-1 (nuclear receptor/transcription factor), have also been implicated in the regulation of cancer stem cell self-renewal. In cancer stem cells, these pathways are believed to be deregulated, leading to uncontrolled self-renewal of cancer stem cells which generate tumors that are resistant to conventional therapies. Current cancer therapeutics based on tumor regression may target and kill differentiated tumor cells, which compose the bulk of the tumor, while sparing the rare cancer stem cell population. The cancer stem cell model suggests that the design of new cancer therapeutics may require the targeting and elimination of cancer stem cells. Therefore, it is imperative to design new strategies based upon a better understanding of the signaling pathways that control aspects of self-renewal and survival in cancer stem cells in order to identify novel therapeutic targets in these cells. CSC-directed therapeutic approaches might represent translationally relevant strategies to improve clinical cancer therapy, in particular for those malignancies that are currently refractory to conventional anticancer agents directed predominantly at tumor bulk populations.

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