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CBP/Catenin Antagonists: Targeting LSC's Achilles Heel

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Abstract

Cancer Stem Cells (CSCs) including Leukemia Stem Cells (LSCs) exhibit self-renewal capacity and differentiation potential, and have the capacity to maintain or renew and propagate a tumor/leukemia. The initial isolation of CSCs/LSCs was in adult myelogenous leukemia, although more recently, the existence of CSCs in a wide variety of other cancers has been demonstrated. CSCs in general, and LSCs specifically in regards to this review, are responsible for initiation of disease, therapeutic resistance and ultimately disease relapse. One key focus in cancer research over the past decade has been to develop therapies to safely eliminate the LSC/CSC population. One major obstacle to this goal is the identification of key mechanisms that distinguish LSCs from normal endogenous hematopoietic stem cells. An additional daunting feature that has recently come to light with advances in next generation sequencing and single cell sequencing is the heterogeneity within leukemias/tumors, with multiple combinations of mutations, gain and loss of function of genes, etc. being capable of driving disease, even within the CSC/LSC population. The focus of this review/perspective will be on our work in identifying and validating in both CML and ALL, a safe and efficacious mechanism to target an evolutionarily conserved signaling nexus, which constitutes a common "Achilles Heel" for LSC/CSC, utilizing small molecule specific CBP/catenin antagonists.

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1. Introduction

Stem cells are cells that by definition possess both the capability to self-renew (i.e. give rise to at least one identical daughter cell) as well as differentiate into more mature, specialized cell types. Stem cells can be pluripotent, embryonic stem cells ES or induced pluripotent stem cells (iPS), or of adult tissue origin, termed somatic stem cells (SSC). Somatic stem cells have undergone a partial differentiation process, restricting their differentiation potential, and are hence termed multi-, oligo- or bipotent (1;2). Throughout our lifetime, long-lived, essentially "immortal", somatic stem cells are called upon to renew and regenerate adult tissues both during homeostatic processes and repair after insult or injury. However, with aging, there is a significant deterioration in stem cell function in a wide array of tissues including blood (lymphoid lineage decreases, myeloid lineage increases and erythroid lineage decreases) (3), which is also associated with increased cancer risk (4). The first type of SSC to be isolated and utilized therapeutically was the hematopoietic stem cell (HSC) in the form of bone marrow for transplantation therapy (5). The dark side of the immortality of SSCs/HSCs is their capacity to be corrupted thereby generating cancer stem cells (CSCs) including leukemia stem cells (LSCs). Like their normal counterparts, CSCs/LSCs exhibit self-renewal capacity and differentiation potential, albeit with aberrant and incomplete differentiation potential, and have the capacity to maintain or renew and propagate a tumor/leukemia. The initial isolation of CSCs/LSCs was in adult myelogenous leukemia (AML)(6), although more recently, the existence of CSCs in a wide variety of other cancers has been demonstrated(7). CSCs in general and specifically in regards to this review, LSCs, are responsible for initiation of disease, therapeutic resistance and ultimately disease relapse (8).

Consequently, one key focus in cancer research over the past decade has been to develop therapies to safely eliminate the CSC/LSC population. A major obstacle to this goal is the identification of key mechanisms that distinguish LSCs from normal endogenous hematopoietic stem cells (HSCs). One additional daunting feature has come to light with recent advances in next generation sequencing and single cell sequencing. It is now clear that cancer is an extremely heterogeneous disease with multiple combinations of mutations, gain and loss of function of genes, etc. being capable of driving disease. Furthermore, within an individual tumor and even within the CSC/LSC population in the tumor, heterogeneity will be a significant problem to overcome (9–11). The focus of this review/perspective will be on our pre-clinical and translational studies in identifying and validating in both CML and ALL, a safe and efficacious mechanism to target the LSC population via a common "Achilles Heel".

2. Hematopoietic Stem Cells versus Leukemic Stem Cells; More Alike than Different

Unfortunately, from the standpoint of safely targeting LSCs, it appears that the similarities between normal HSCs and LSCs far outweigh the differences between them. (For a recent additional perspective on this topic please see Koeffler and Leong (21)).

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This is not all that surprising in that LSCs, in many instances, likely arise from HSCs via mutations (12;13). Importantly, by the definition of "stemness", they both possess the ability to self-renew and also proceed on to more differentiated cell types. LSCs express similar "stemness" markers and exhibit cellular behaviors highly reminiscent of HSCs. LSCs and HSCs appear to co-inhabit the same specialized niches in the bone marrow and in fact can compete with one another for the limited space within the niche (14–17). Long-lived HSCs are relatively quiescent, infrequently entering cell cycle to maintain homeostasis but more frequently upon injury to repair damaged tissue. Similarly, LSCs appear to be generally quiescent (18). The same signaling pathways involved in regulating LSCs (i.e., Wnt, Notch, Hedeghog, TGFβ/BMP, JAK/Stat, Hippo, MAPK/PI3K) are also involved in the regulation of HSCs (19;20) and multiple points of intersection and crosstalk, including feedback and feedforward loops, connect the various signaling cascades that modulate "stemness".

3. Wnt Signaling and Stemness

Wnt signaling constitutes an ancient pathway dating back to the early metazoans. The Wnt/catenin pathway is critical throughout normal embryonic development and the life of the organism. It is an extremely complex signal transduction pathway involving 19 mammalian Wnt ligands (22) that trigger a variety of intracellular responses broadly classified as either canonical (increase in nuclear β-catenin) or noncanonical (planar cell polarity, $Ca^{2+/}$ PKC activation) (23;24). The former is often associated with proliferation and lack of differentiation (for example, as a hallmark of dysregulated Wnt signaling in cancer), whereas the latter is often associated with cell, tissue, and organ differentiation. However, this really is a gross oversimplification (for recent reviews please see (25–27)). β-catenin through its nuclear functions and cytoskeletal/cytoplasmic membrane interactions plays important roles in both canonical and non-canonical Wnt signaling, respectively. In reality, a continuum exists, that coordinates β- catenin-dependent gene expression and cytoplasmic/cytoskeletal β-catenin to affect key developmental and regulatory processes. The entry of β-catenin, or other catenins, for example γ- catenin/plakoglobin (28), into the nucleus and subsequent transcriptional processes are controlled by the so-termed canonical Wnt or Wnt/β-catenin signaling cascade. However, alternative signaling cascades also induce the nuclear translocation of β-catenin and its subsequent participation in transcription. For example, receptor tyrosine kinases (29) and non-receptor tyrosine kinases including Src(30) and Abl(31) can enhance β-catenin-mediated transcription. Additionally, prostaglandins (32), hypoxia (33;34), and high glucose levels (35), also activate Wnt/catenin signaling. These signals are integrated with signals from other key pathways including Notch, JAK/Stat etc., providing nuclear β-catenin with an essential role in balancing self-renewal versus differentiation in adult stem cells (36;37). Wnt signaling is clearly critical in stem cell biology; however, there is no consensus as to whether Wnt signaling is important for either maintenance of potency (8;38) or the differentiation of stem cells (39). Wnt/β-catenin signaling clearly plays dichotomous roles in stem cell biology (38–40).

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4. Wnt/Catenin Signaling, Hematopoiesis and HSC, Leukemia and LSC: Differential Coactivator Usage

Wnt signaling, both the canonical and noncanonical pathways, play important roles in hematopoiesis. Retroviral overexpression of activated β-catenin expands the pool of HSCs in long-term cultures and these HSCs activate a LEF-1/TCF reporter in their normal *in vivo* microenvironment. Inhibitors of the Wnt signaling pathway, as well as ectopic expression of axin or a frizzled ligand-binding domain, results in reduction of HSC growth *in vitro* and diminished reconstitution *in vivo* (41).

Genetic deletion of β-catenin during fetal development leads to the impairment of HSC self-renewal. However, several reports have indicated that adult HSCs do not require β-catenin for maintenance (42) and that canonical Wnt signaling regulates hematopoiesis in a dose-dependent fashion (43). Armstrong and coworkers demonstrated that although deletion of β-catenin after CML initiation does not lead to a significant increase in survival, deletion of β-catenin synergizes with imatinib to delay disease recurrence after termination of imatinib treatment. Pharmacologic inhibition of β-catenin using the cyclooxygenase inhibitor indomethacin reduces β-catenin levels and leads to a reduction in LSCs (44). Aberrant Wnt activation need not be cell intrinsic however, as constitutively active β-catenin in osteoblasts is a driver of acute myeloid leukemia (AML) (45). Interestingly, the development of BCR-ABL positive B-ALL and the *in vivo* self-renewal of B-ALL LSCs were apparently not affected by the absence of β-catenin (46). Furthermore, hematopoiesis occurred normally in the combined absence of β- and γ-catenin using double β/γ-catenin knockout mice (42;47). However, Wnt signaling as judged by Axin2 expression is still maintained and the HSCs maintain long-term repopulation capacity and multilineage differentiation potential, thus pointing to an alternative 'catenin-like molecule compensating for the loss of both beta and gamma catenin (48).

The Wnt pathway has emerged as a pivotal player in the specification and maintenance of SSC in multiple stem cell niches, in a wide array of tissues and organs including the hematopoietic system (49). It is therefore not surprising that aberrant regulation of Wnt signaling is a recurrent theme in cancer (50;51). This has engendered significant effort to develop therapeutic approaches to target Wnt signaling. A number of factors have thwarted progress in this field, including the enormous complexity of the pathway (23). Further complexity is encountered when targeting transcriptionally active β-catenin, as β-catenin, as well as other catenins (e.g. γ-catenin (52)) can bind a broad spectrum of transcription factors outside of classical Wnt signaling partners, i.e. members of the TCF/LEF family (53). Transcriptionally active β-catenin is associated with an array of biological processes including maintenance of potency, EMT, oxidative stress, and lineage commitment (53). Successful therapeutic manipulation of endogenous "stemness" (normal or cancerous) via modulation of aberrant catenin-regulated transcription offers enormous promise, however it requires significant precision to prevent deleterious effects (e.g., depletion of or increases in somatic mutations) in normal SSC populations (54).

Aberrant Wnt/β- and γ-catenin signaling has been associated with the development of AML (55;56) as well as a critical pathway in the self-renewal of CML LSCs(57;58). Furthermore,

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transduction of γ-catenin into primitive hematopoietic progenitor cells preserved their immature phenotype during colony formation, suggesting enhanced self-renewal capacity and γ-catenin-transduced cells accelerated the development of leukemia in syngeneic mice (56). However, loss of both β- and γ-catenin leaves Wnt signaling, hematopoiesis and lymphopoiesis intact (59), pointing to yet uncharacterized catenin-like molecule(s) that can compensate for the loss of both β- and γ-catenin.

β- (or γ-)catenin recruits one of the two Kat3 transcriptional coactivators, cAMP response element binding protein (CREB-binding protein (CBP) or its closely related homolog, p300 (E1A–binding protein, 300 KDa) as well as other components of the basal transcriptional apparatus to generate a transcriptionally active complex (24;60) (Fig. 1a). The Kat3 coactivator family, CBP and p300, diverged via gene duplication approximately 450 million years ago. These Kat3 coactivators interact with hundreds of proteins in their roles as master orchestrators of transcription. Due to their high degree of protein sequence identity and even higher similarity, they have long been considered largely redundant. However, accumulating evidence has demonstrated that CBP and p300 are not redundant and play definitive and unique roles in vertebrate biology (61–65). Seventeen years ago, from a library of secondary structure mimetics, our lab identified ICG-001 in a forward chemogenomic screen. We subsequently demonstrated that ICG-001 binds specifically and with high affinity (~1 nM) to the N-terminus of CBP (66;67). Over the years, we found that ICG-001, via selectively blocking the CBP/catenin interaction leads to the initiation of differentiation programs in a wide array of stem/progenitor cells (ES, iPS, and SSC) (68;69) (Fig. 1b). Further investigations led to our model of differential coactivator usage, which highlights the distinct roles of the coactivators CBP and p300 in catenin-mediated transcription, particularly within stem/progenitor populations (Fig. 1)(70).Differential utilization of either CBP or p300 as the catenin coactivator is the first decision that guides a stem cell to either maintain potency or initiate a differentiative transcriptional program, respectively (Fig. 1a). We have subsequently identified several small molecules (IQ-1, ID-8, and the specific direct p300/catenin antagonists YH249/250) that selectively antagonize the p300/catenin interaction, thereby enhancing the CBP/catenin interaction, resulting in enhancement of symmetric divisions and the maintenance of potency (pluri- or multipotency) in a variety of stem cell populations (ES, iPS, SSC and HSC) (68;70–73) (Fig. 1c).

5. Wnt Signaling in CML

Despite the stunning clinical success achieved treating chronic-phase (CP) CML patients, responses in advanced-phase patients treated with the TKI Gleevec/Imatinib (IM) are often short-lived, and patients generally undergo disease progression (74). Furthermore, resistance to IM develops in 2–4% of patients annually and IM dose escalation is generally not effective (75).The insensitivity of quiescent CML stem cells to TKIs that express low levels of *bcr-abl*, has been proposed as a mechanism of resistance(76). Increased nuclear β-catenin has been observed with progression to blast crisis (BC) (77;78). Leukemia stem cells (LSC) are insensitive to TKIs, and additionally, genomic instability in this subpopulation is a significant concern (79). Epigenetic silencing of negative regulators of the Wnt signaling cascade is also frequently observed in leukemias, including CML (80). Chromosomal aberrations (81), alterations in the bone marrow microenvironment (82), as well as other

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mechanisms, may play significant roles in LSC resistance (for a recent review on CML LSC resistance please see T. Holyoake and D. Vetrie (83)). β-Catenin signaling has also been reported to be activated during the development of MLL (mixed-lineage leukemia) leukemic stem cells(84).

6. Wnt Signaling in ALL

Despite significant progress over the past decades, drug resistance remains a major problem in the treatment of ALL. Dose escalation of current chemotherapeutics is limited by acute and chronic toxicity; therefore new treatment modalities are required. Aberrant Wnt/catenin signaling has been reported to play critical roles in both AML (55) and CML (57;58), where leukemic drug resistant clones have been associated with increased nuclear β-catenin levels (85). However, less is known about the role of Wnt signaling in ALL. Wnt3a has been shown to drive the proliferation of the precursor B-ALL cell lines NALM6, REH and LK63 (86) and endogenous WNT16b expression has been found to be upregulated by the TCF3-PBX1 (E2A–PBX1) fusion (87) . Furthermore, siRNA knockdown of WNT16b, thereby decreasing canonical Wnt/β-catenin signaling, has been shown to initiate apoptosis and reduces the expression of the Wnt-regulated target gene *survivin* (*BIRC5*) (87), which has been implicated in both leukemia cell survival and drug resistance (88–90).

7. Wnt signaling in T-ALL

Wnt signaling has been identified as one of the important self-renewal pathways in T-ALL (91;92), Constitutive Wnt/β-catenin signaling was shown to lead to T-ALL in mice (93) . Leukemia stem cells from *Pten*^{null} mouse T-ALLs have been shown to have increased levels of β-catenin protein (94) and a real-time, integrated fluorescent Wnt reporter was shown to mark rare leukemia stem cells in T-ALL (95). Over 85% of childhood T-ALL patients showed upregulated β-catenin expression and upregulation of the Wnt target genes *axin2, c-myc, tcfl* and *lef* (96). Silencing of β-catenin by small interfering RNA led to increased apoptosis (96). Wnt inhibition by transduction of lentivirus encoding dnTCF into human T-ALL cell lines (HPBALL and RPMI 8402) led to survival prolongation in xenografted T-ALL in mice. Treatment with the tankyrase inhibitor XAV-939 also led to decreased proliferation *in vitro* (95).

8. Stem Cell Decisions: Symmetry versus Asymmetry

Long-lived HSCs remain relatively quiescent for the majority of their lifetime during normal tissue homeostasis, perhaps dividing only once every few months (97) or even less frequently (98). HSCs can divide either symmetrically or asymmetrically (Fig. 2). Ideally, an asymmetric balance is maintained, whereby one of the daughter cells remains in its niche as a stem cell, while the other daughter proceeds to initiate the differentiation process to maintain tissue homeostasis (Fig. 2a). However, this asymmetric balance is not always maintained and HSCs can also undergo symmetric divisions. There are two modes of symmetric division. In symmetric non-differentiative divisions, both daughter cells remain as stem cells in the niche. Alternatively, HSCs can undergo symmetric differentiative divisions, where both cells leave the niche and go on to differentiate, thereby losing their

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"stemness" (Fig. 2b). Both modes of symmetric division are presumed to be deleterious to the normal long-lived HSC population, as they can either lead to premature exhaustion of the HSC pool (via symmetric differentiative divisions) or alternatively increase the number of DNA mutations accumulated in the HSC pool (via symmetric non-differentiative divisions). More than 40 years ago, Cairn's "immortal strand hypothesis" provides a potential, although still controversial, rationale for the preference of HSCs to undergo asymmetric versus symmetric cell divisions (99).

Although symmetry versus asymmetry is essentially a simple binary decision process, a HSC/LSC located in its niche undergoing mitosis must read an enormously complex array of information from its environment including oxygen levels, nutrient levels, circadian cycles, nervous system innervation, growth factors, adhesion molecules, kinase cascades, cell–cell contacts, etc. to arrive at this eventual binary decision. Interestingly, a bias towards symmetric over asymmetric divisions appears to be a key fundamental difference between LSCs and HSCs. For example, loss of function of the tumor suppressor PTEN leads to premature exhaustion of the normal HSC population (presumably due to increased symmetric differentiative divisions), whereas there is an expansion of the LSC population (presumably due to increased symmetric non-differentiative divisions)(100).More generally, the decision to preferentially undergo symmetric non-differentiative versus symmetric differentiative divisions appears to be an intrinsic difference between cancer stem cells (CSCs) and normal somatic stem cells (SSCs) carrying critical mutations in a number of pathways (i.e., p53, p73, PTEN, Hedgehog, Notch etc.) (101;102). This provides a potential mechanism to attempt to stochastically eliminate mutated defective SSCs prior to the accumulation of additional deleterious mutations (63). A stem cell's decision to enter cycle and divide symmetrically or asymmetrically or to remain quiescent is clearly governed by the integration of multiple signaling cascades. The key is to understand how this diverse array of signals and crosstalk are integrated and processed into the simple, yet critical decision to divide symmetrically or asymmetrically.

9. Pharmacologically Manipulating HSC and LSC

Over the past 15 years, we have examined the therapeutic potential of selectively antagonizing the CBP/catenin interaction in a variety of preclinical tumor models (both solid and liquid tumors). During the course of our investigations, we observed that CBP/catenin antagonists (i.e. ICG-001), in conjunction with standard chemotherapeutic agents (targeted or cytotoxic agents), demonstrated the ability to safely eliminate drug-resistant CSCs via forced differentiation, without deleterious effects on the normal endogenous stem cell populations and furthermore, ameliorated the toxicity of standard regimens. The differential effects of CBP/catenin antagonists on LSC versus normal HSC and more generally SSC (i.e., forced differentiation and elimination versus differentiation and enhanced repair without depletion) must therefore be cell intrinsic.

CBP/catenin antagonists apparently take advantage of the intrinsic propensity of LSCs to increase the number of symmetric divisions at the expense of asymmetric divisions due to various mutations (e.g., p53, PTEN, etc.) (100;101). Normal long-term repopulating HSCs preferentially divide asymmetrically with one daughter cell remaining in the niche and the

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other going on to a transient amplifying cell required for hematopoiesis (31), whereas LSCs undergo more symmetric division. We proposed that this fundamental cell intrinsic difference between HSCs and LSCs provides a unique opportunity to therapeutically target and eliminate LSCs without damaging the normal endogenous HSC populations via CBP/catenin antagonists forcing symmetric differentiative divisions in LSCs, while the normal HSCs asymmetrically divide (71;103;104).

10. Targeting CML LSC

The ability to safely eliminate the drug-resistant LSC population, without damaging endogenous HSCs, is critical to the development of more effective chemotherapeutic strategies that completely eliminate the leukemia. We demonstrated that ICG-001, by specifically antagonizing the interaction between CBP and catenin (both β and γ) in CML, initiates a differentiative pathway. This is manifested in the increased expression of myeloid and megakaryocyte differentiation markers including CD11b, CD16, CD33, CD56 and CD41.CBP/catenin antagonists are differentiating agents and not *per se* cytotoxic, therefore only limited apoptosis is observed. However, the forced differentiation of LSC leads to decreased expression of the antiapoptotic gene *survivin*, which we have previously shown to be CBP/catenin dependent (61;88), with a concomitant increase in the expression of oncoprotein BCR-ABL. These effects on LSCs are associated with elimination of the quiescent LSC population via forced differentiation into the 'bulk' CML population that is sensitive to BCR-ABL antagonists. Downregulation of survivin expression appears to be specific to LSCs (and more generally CSCs). Interestingly, CBP/catenin antagonists do not appear to cause a reduction in survivin expression in normal HSCs or other tissues (epidermal stem cells for example) *in vivo* based upon the fact that no deleterious effects on these populations have been observed after long term (up to 2 years in mice) administration of CBP/catenin antagonists.

Pretreatment of CML cells *in vitro* with ICG-001, although not killing the leukemia cells, eliminated, essentially irreversibly, the LSC population, as judged by the lack of engraftment into NSG mice. Importantly, ICG-001 in combination with the second generation TKI Nilotinib, safely eliminated engrafted K562 CML cells as well as primary CML patient samples, in highly immunocompromised NSG mice, without any apparent deleterious effects to the normal endogenous HSC population, as judged by normal hematopoietic parameters and a normal life span (60).

11. Targeting ALL

Sequence or deletion mutations of CBP have recently been identified in ALL (105;106).Extensive analysis of an extended cohort of 71 ALL relapse patients with 270 cases that did not relapse found that 18.3% of relapse cases had sequence or deletion mutations of CBP. In addition, inactivating CBP mutations have been described as a common event in follicular lymphoma and diffuse large B-cell lymphoma (107), the two most frequent forms of B-cell Non-Hodgkin's lymphoma. Interestingly, of the hundreds of samples sequenced, most mutations occurred within the histone acetyl transferase (HAT) domain with only one described within the N-terminus of CBP, which constitutes both the

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catenin and ICG-001-binding site. We previously proposed that based upon the critical role for the CBP N-terminal/catenin interaction in maintaining the LSC population, mutations in this N-terminal region would generally not be selected for. Importantly from a therapeutic standpoint, we demonstrated that ICG-001 can also sensitize B-ALL cell lines harboring CBP HAT domain mutations to chemotherapy (104).

The unifying fundamental therapeutic concept therefore, is that in both CML and ALL, CBP/catenin antagonism can deplete drug-resistant LSCs by interruption of self-renewal and shift of catenin/coactivator function (88;90). Antagonizing the CBP/catenin interaction forces symmetric differentiation and additionally down-regulates *survivin* expression in LSC, thereby sensitizing the cells to chemotherapy, without depletion or deleterious effects on the normal HSCs that undergo asymmetric differentiation. We demonstrated the abrogation of self-renewal by inhibition of serial re-plating of primary ALL cells after treatment with ICG-001. Furthermore, ICG-001 induced, in a dose-dependent manner, the differentiation of murine BCR- ABL1-transformed pre-B cells (CD19+B220+) as determined by analysis of κ-light chain surface expression, a hallmark of B-cell differentiation (104).

12. To the Clinic

In principle, significant concerns about specificity and thereby off-target toxicity, could be raised concerning small molecule inhibitors that target the coactivator protein CBP, as CBP has as many as 500 molecular partners, including a vast array of transcription factors (108). However, these concerns have not been borne out either pre-clinically or even more importantly clinically, utilizing either ICG-001 or the second-generation clinical CBP/ catenin antagonist PRI-724(109). This is perhaps at first very surprising. The extremely high biochemical selectivity of ICG- 001/PRI-724 for the N-terminus of its molecular target CBP, the fact that these agents only disrupt a small subset of total CBP interactions and finally the unique evolutionarily conserved roles of the two Kat3 coactivators CBP and p300(63),can be used to rationalize the safety of these agents.

The second-generation specific CBP/catenin antagonistPRI-724 (IC50 ~150 nM) developed by Prism Pharma was safe in preclinical IND enabling toxicology studies, with the no adverse event level being 120 mg/kg/day in dogs given by a 28-day continuous infusion. PRI-724 is in the clinic for both solid tumors (colorectal and pancreatic) and hematopoietic malignancies (CML and AML). An additional trial for HCV induced hepatic fibrosis was also initiated.

An open label, phase Ia safety study in subjects with solid tumors was conducted at USC and reported at ASCO in June 2013 (109). PRI-724 had a very acceptable toxicity profile with dose escalation from 40 to 1280 mg/m2/day with 7 days of continuous i.v. infusion. Downregulation of the biomarker *survivin/BIRC5* with upregulation of the differentiation antigen *CK20* in CTCs (circulating tumor cells) strongly correlated with increasing plasma concentrations of drug in colorectal cancer patients (109). Additional oncology trials and a trial for HCV-induced hepatic fibrosis with PRI-724 were subsequently initiated (https://clinicaltrials.gov). An open-label, dose-escalation phase Ib/IIa study of PRI-724 for

advanced myeloid leukemia at MD Anderson and other cancer centers further demonstrated that PRI-724 is well tolerated. A mechanistic evaluation of patient samples showed that PRI-724 treatment downregulated the expression of the Wnt/catenin targets CD44 and survivin (110).

13. Perspective: CBP/catenin Antagonists: Targeting LSC's Achilles Heel

As noted in the introduction, tumor heterogeneity and the ability to safely target the drug resistant CSC/LSC population are two common and extremely vexing problems that we must be able to overcome before we can dramatically change overall outcome when treating malignancies. In this respect, CBP/catenin antagonists appear to be truly unique in their ability to target LSCs and more generally drug-resistant CSCs (111;112) in a wide range of tumors that seemingly have little in common with respect to mutational drivers, mechanisms associated with malignancy, i.e. genetic mutations or epigenetic changes, or cell of origin. Even more importantly, CBP/catenin antagonists do so without damaging the normal endogenous stem cell population (71). What provides CBP/catenin antagonists with this unique profile? Quiescence provides long-lived, essentially immortal, HSCs and more generally SSCs, with a safeguard to preserve their functionality by limiting damage to the cell caused by mitochondrial oxidative phosphorylation, DNA damage, and uncontrolled cell cycle entry and exhaustion of the stem cell pool via symmetric differentiative divisions (113;114). HSCs, as well as LSCs, prefer glycolysis rather than oxidative phosphorylation despite the inefficiency in regards to ATP generation (115). The switch from glycolysis to oxidative phosphorylation is associated with activation of quiescent HSCs and the initiation of differentiation (116).

Roughly 450 million years ago, the evolution of vertebrates initiated a new lifestyle requiring critical adaptations, including long-term homeostatic maintenance and tissue repair. This necessitated the advent of SSCs and their corresponding niches, to maintain a relatively quiescent anaerobic metabolic state as opposed to their more proliferative aerobic-differentiated daughter cells, in order to protect the integrity of the genetic material in the stem cell pool (117). This further required a robust, high fidelity mechanism to ensure the proper maintenance of "stemness" in one daughter cell, while in the other daughter cell allowing the initiation of a differentiative program. Intriguingly, the Kat3 coactivator family CBP and p300 diverged via gene duplication apparently just prior to the vertebrate radiation over 450 million years ago. CBP and p300 are extremely large proteins encoded over 33 and 31 exons respectively. Despite having diverged over 450 million years, CBP and p300 retain an extremely high degree of identity, up to 93 %, particularly over a large central core that includes the CH1, KIX, Bromodomain, and CH2 and CH3 regions (Fig. 3)(118;119). Interestingly, the least conserved region, with only 66 % identity between the two Kat3 coactivators, is the extreme N-terminal region, to which both β- (and γ) catenin and the small molecules ICG-001/PRI-724 bind. Yet, the N-terminal regions within each orthologous group are highly conserved for at least the past ~100 million years of evolution; for example, human and mouse CBP are 98 % identical at the amino acid level within this region. One would also assume that over millions of years of evolution that "naturally occurring" CBP/catenin antagonists would have evolved that could assure the asymmetric differentiation of the long lived, highly quiescent SSC pool. Interestingly, the very amino-

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termini of both CBP and p300 appear to be a nexus for the integration of many signal transduction pathways. For example, a highly conserved LXXLL sequence is present in this region of both CBP and p300, which can recruit nuclear receptor signaling complexes to this region of the Kat3 coactivators. In that sense, we believe that there are numerous naturally occurring CBP/catenin antagonists. For example, all-trans retinoic acid (ATRA) is very effective for the treatment of Acute Promyelocytic Leukemia (APL). Similarly to ICG-001, ATRA does not kill the malignant cells but induces them to differentiate. Vitamin D occupies a prominent position in cancer prevention. Both ATRA and vitamin D, via their respective transcriptional complexes (RAR/RXR and VDR/RXR), can antagonize aberrant Wnt signaling (120) and thereby phenocopy ICG-001/PRI-724, via competition for catenin binding to the N-terminus of CBP.

However, synergistic effects on the activation of gene expression by ATRA and Wnt for example (Szeto et al. 2001), have been reported and both vitamin D and ATRA drive the expression of distinct cassettes of genes. We have recently proposed (63) and subsequently have confirmed (*Ono et al manuscript submitted*) that a highly evolutionarily conserved 27bp deletion in CBP, between the β-catenin-binding region and the LXXLL nuclear receptor binding sequence regulates nuclear receptor antagonism or synergy with Wnt/catenin signaling. ATRA and vitamin D therefore are not simply “pure antagonists” of CBP/catenin signaling and, in that sense, differ from ICG-001/PRI-724. Interestingly, a number of nuclear receptors, both ligand and “orphan” receptors, also demonstrate the ability to either maintain potency or initiate differentiation in stem cell populations, in a similar manner to what we have observed with specific CBP/catenin or p300/catenin antagonists (73;121). For example, PPARδ agonists improved HSC maintenance via increased asymmetric division presumably via CBP/catenin antagonism (122). In that sense, nuclear receptor ligands can behave as CBP/catenin antagonists partially phenocopying ICG-001/PRI-724. However, there are several important differences. Small molecule CBP/catenin antagonists are direct inhibitors (i.e., they bind directly to CBP and do not require any protein cofactors e.g. RAR/RXR) and are pure CBP antagonists (i.e., they have no agonistic activity *per se*). Furthermore, they allow for stochastic differentiation (i.e., non-deterministic), whereas ATRA or vitamin D, after antagonizing the CBP/catenin interaction, via p300-dependent agonistic properties, bias lineage commitment. We have previously proposed that this p300-dependent lineage biasing is associated with the deleterious effects of high concentrations of ATRA on embryonic development (123), which are not observed in mice treated with ICG-001 *in utero* (63). Striking differential coactivator usage by the nuclear receptor family has also observed in prostate cancer cells, where 47 % of androgen-regulated genes were p300-dependent, whereas only 0.3 % was CBP-responsive (124). Beyond conserved catenin and nuclear receptor binding regions, the interferon responsive transcription factor Stat1 has also been shown to bind to the very amino terminus of CBP and p300. Further, there are approximately 20 serine and threonine residues that can be post-translationally modified within the first 111 amino acid residues of CBP and p300 (125). In our view, in this fashion the N-termini of the Kat3 coactivators function as a nexus for the integration of an array of signaling cascades that determine the critical stem cell decision to divide symmetrically or asymmetrically, via controlling the balance between the CBP/catenin and the p300/catenin interaction (63). We propose, therefore, that CBP/catenin antagonists can safely target a

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common "Achilles Heel" in LSC in both CML and ALL and more generally CSCs in other malignancies. CBP/catenin antagonists by specifically and directly binding to CBP initiate differentiation in both LSC and HSC. However, CBP/catenin antagonists take advantage of the preference of LSCs to divide symmetrically relative to asymmetrically, thereby CBP/catenin antagonists via forced differentiation, stochastically "differentiate away" LSC from their niche to more differentiated bulk leukemia and thus targetable by conventional chemotherapy (i.e. Imatinib or cytotoxic agents). However, HSC preferentially divide asymmetrically, thereby always maintaining one stem cell in the niche. Therefore CBP/catenin antagonists do not deplete the endogenous normal HSC pool. Furthermore, by targeting the highly conserved N-terminus of CBP, that appears to be relatively devoid of escape mutations, likely due to its critical role in stem cell maintenance, resistance to CBP/catenin antagonists appears to be quite unusual. Finally, the role of the N-terminus of CBP as a signaling nexus allows CBP/catenin antagonists to work effectively against an enormous range of mutations (p53, PTEN, KRAS, BRAF, APC etc.), signaling networks and epigenetic modifications. The fundamental nature of this balance of coactivator usage by catenin is already manifested at the first cellular decision point in mammalian biology (i.e. at the 8 cell stage of embryogenesis) and we proposed that it is carried through all stem cell populations in vertebrates (63). Thus the ability to target LSCs in ALL and CML, we believe to be just a glimpse of the capacity of CBP/catenin antagonists to safely treat malignancies via elimination of drug-resistant CSCs to provide real cures for these malignancies, similar in this regard to the efficacy of bone marrow transplantation. Further clinical investigation will be needed to confirm this.

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Highlights

- Wnt signaling regulates self-renewal and differentiation in normal stem cells.
- Aberrant Wnt signaling in leukemia stem cells (LSC) is associated with resistance.
- The small molecule ICG-001 selectively blocks the CBP/β- or γ-catenin interaction.
- CBP/catenin antagonists may safely target an “Achilles Heel” in LSC.

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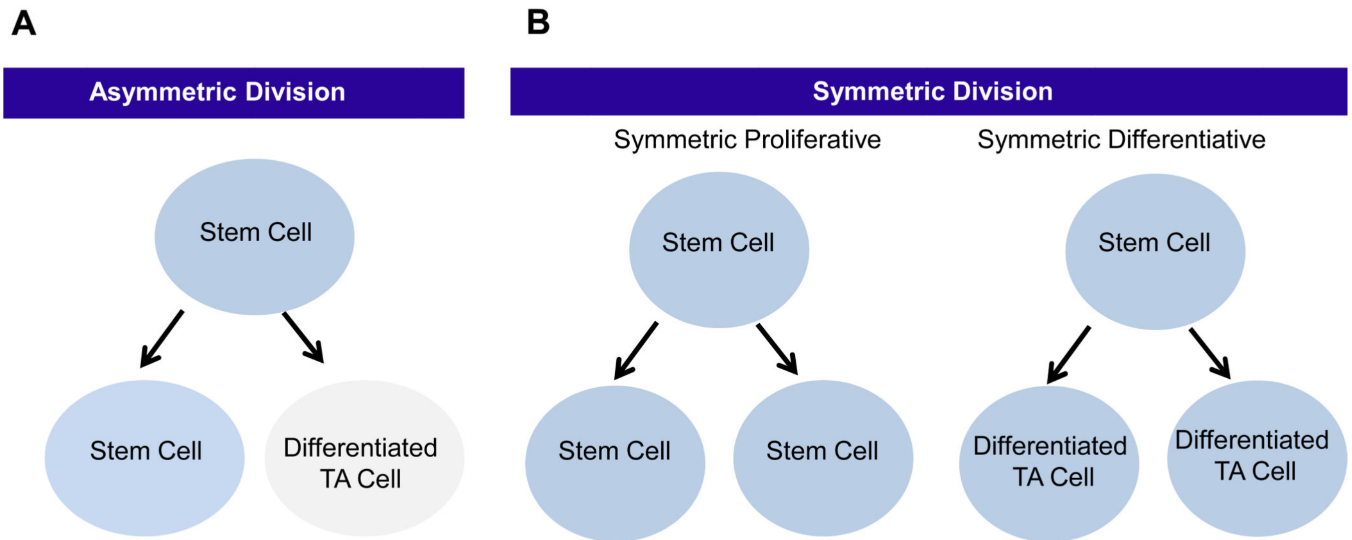

Figure 1.

(A) An asymmetric division results in the production of two daughter cells with different cell fates – one a stem cell and the other a differentiated transient amplifying (TA) cell.

(B) A symmetric proliferative division occurs when the two daughter cells remain as stem cells. A symmetric differentiative division gives rise to two daughter cells, both of which are differentiated TA cells.

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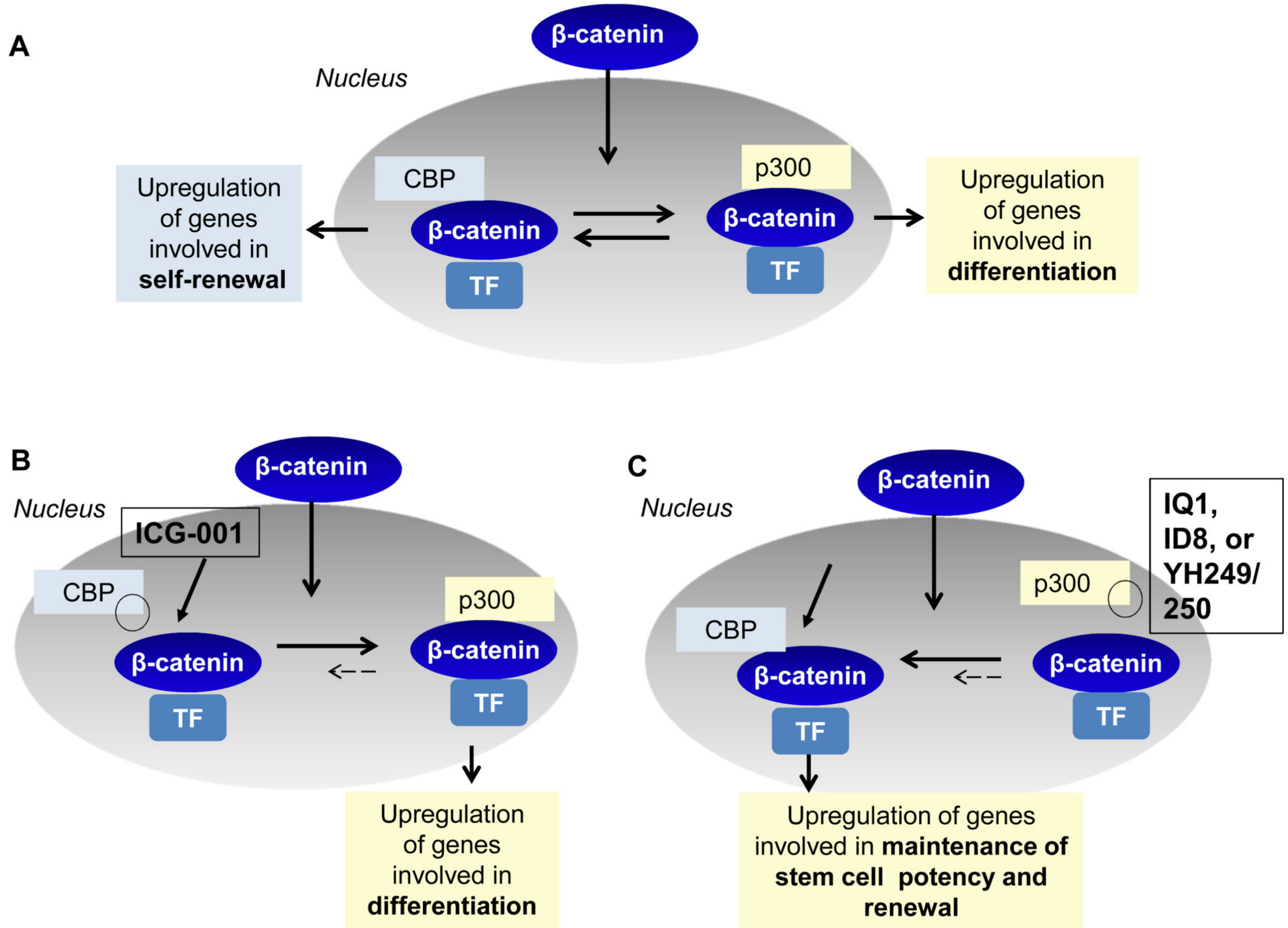

Figure 2.

(A) Upon translocation into the nucleus, β-catenin can associate with the co-factors CBP upregulating ultimately genes involved in self-renewal, or p300 and thereby genes involved in the initiation of differentiation of the CSC/LSC population.

(B) ICG-001 selectively blocks the interaction between β-catenin and CBP. This results in biasing towards p300 usage, and thereby initiates the differentiation transcriptional program with the loss of self-renewal capacity of CSC/LSC.

(C) IQ-1, ID8 (both indirectly) and YH 249/250 (directly) block the interaction between β-catenin and p300. By selectively blocking this interaction, CBP usage is increased, and consequently the initiation of a proliferative or self-renewal transcriptional program is favored.

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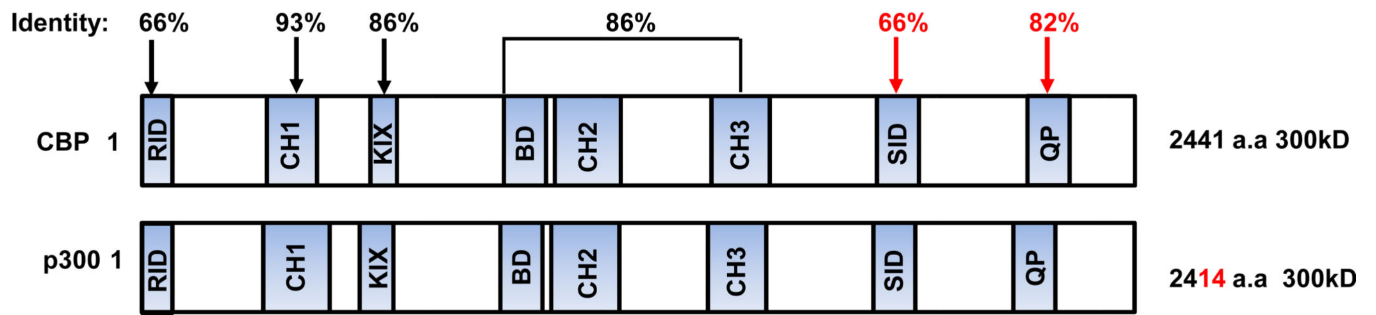

Figure 3.

Schematic representation of CBP and p300 and the high percentage of identity at the amino acid level between various regions of these largeKAT3 coactivators.