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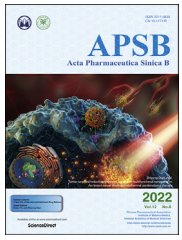

REVIEW

Development and clinical advancement of small molecules for *ex vivo* expansion of hematopoietic stem cell

Jiaxing Li[a,b,†], Xiao Wang[a,b,†], Jiayu Ding[a,b], Yasheng Zhu[a,b], Wenjian Min[a,b], Wenbing Kuang[a,b], Kai Yuan[a,b], Chengliang Sun[a,b], Peng Yang[a,b,*]

[a]*State Key Laboratory of Natural Medicines and Jiang Su Key Laboratory of Drug Design and Optimization, China Pharmaceutical University, Nanjing 210009, China*
[b]*Department of Medicinal Chemistry, School of Pharmacy, China Pharmaceutical University, Nanjing 210009, China*

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Abstract Hematopoietic stem cell (HSC) transplantation is the only curative therapy for many diseases. HSCs from umbilical cord blood (UCB) source have many advantages over from bone marrow. However, limited HSC dose in a single CB unit restrict its widespread use. Over the past two decades, *ex vivo* HSC expansion with small molecules has been an effective approach for obtaining adequate HSCs. Till now, several small-molecule compounds have entered the phase I/II trials, showing safe and favorable pharmacological profiles. As HSC expansion has become a hot topic over recent years, many newly identified small molecules along with novel biological mechanisms for HSC expansion would help solve this challenging issue. Here, we will give an overview of HSC biology, discovery and medicinal chemistry development of small molecules, natural products targeting for HSC expansion, and their recent clinical progresses, as well as potential protein targets for HSC expansion.

*Corresponding author. Tel: +86 13681986682.
E-mail address: pengyang@cpu.edu.cn (Peng Yang).
[†]These authors made equal contributions to this work.
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1. Introduction

Hematopoietic stem cells (HSCs) are recognized by two exclusive characteristics of self-renewal and multilineage differentiation potential, which make HSC transplantation the only life-saving therapy for many hematologic diseases[1]. HSC transplantation (HSCT) has been widely applied to treat a broad spectrum of hematologic malignancies in clinic, as well as inherited metabolic diseases, autoimmune diseases, sickle cell disease and severe combined immunodeficiency (SCID)[2,3]. To date, although there have been many efficacious drugs to ameliorate hematologic diseases, HSCT is the most efficacious and the only curative therapy that could eradicate hematologic malignancy. There are four main sources for HSCs: bone marrow (BM), mobilized peripheral blood (mPB), placenta blood and human umbilical cord blood. Both bone marrow and mobilized peripheral blood are conventional and most common sources for HSCT therapy. Unfortunately, due to the lack of human leukocyte antigen (HLA)-matched donor, about 30% patients will not have an HLA-matched donor[4]. Furthermore, graft *vs*. host disease (GVHD) remains the most slippery issue that increases the transplant-related mortality of patients receiving mismatched unrelated BM donor.

In recent years, the utilization of UCB has developed rapidly and become an increasingly attractive source for HSC transplantation due to dramatic advantages such as high percentage of HSCs, easy availability, low risk of virus transmission and tolerance of partial HLA-mismatches[5,6]. More importantly, cord blood transplantation is associated with low incidence of chronic GVHD, which threatens long-term survival of patients[5]. However, the lower absolute number of HSCs in a single UCB unit are unmet for adult patients (only 5% CB units provides sufficient cell dose for adults), and restrict UCB to pediatric setting[5,6]. Low number of HSCs in a single UCB unit directly lead to delayed engraftment characterized by longer time to neutrophil and platelet recovery, which results in high risks of infection, transplantation failure and relapse[6]. Recent years, the use of two CB units has improved infusion amount of HSC. However, it shows similar outcomes to a single unit while is associated with a higher risk of GVHD[7]. Since *ex vivo* expansion of HSC is a promising approach to obtain adequate HSC numbers, great efforts have been made to explore feasible methods in clinic during the past two decades. This approach would enable clinical availability of small size and better HLA-matched CB unit.

Since the first UCB transplantation performed in 1988 in a child with Fanconi anemia[6], extensive researches have focused on finding efficacious small molecules for *ex vivo* HSC expansion and great achievements have been made especially in the past decade. Cytokines or growth factors, which have been early intensively investigated to expand HSC, are now essential conditions for culture of HSC expansion. Several small molecules have completed phase I/II clinical trials and some are preparing for phase III trials. Just recently, data of a phase II/III trial of copper chelator tetraethylenepentamine (TEPA) on 101 patients at 25 sites has been reported[8]. To some extent, this is the first well-established small molecule which is also the first small molecule on clinical trial for HSC expansion[9]. Subsequently, more potent and efficacious small molecules SR1, nicotinamide and UM171 were reported in 2010, 2012 and 2014, respectively[10–12]. All of them have been proved safe and feasible in phase I/II trials[5,13,14]. These small molecules usher a new era for *ex vivo* HSC expansion.

Over the past few years, many novel and potent small molecules have been identified, each of which has individual targets and exclusive mode of action. Identification of novel and druggable targets such as YTHDF2[13] and MLLT3[14] not only unveils complex mechanisms of HSC regeneration but also moves forward further discovery of more efficacious small molecules for HSC *ex vivo* expansion.

In this review, we provide an overview of HSC biology and its signaling pathways, and outline small molecules for HSC expansion as well as their activities, mode of action, and clinical progresses. From pharmaceutical chemist's perspective, in order to clarify the medicinal chemistry involved in this field, binding mode, and structure–activity relationship (SAR) studies of several molecules are overviewed, if provided in the literatures. Besides, potential targets for *ex vivo* HSC expansion are also described in the final part.

2. HSC signaling and niche biology

2.1. General characteristic of HSC

Among mammals, HSC emerges and generates in the yolk sac and aorta-gonad-mesonephros (AGM) region, respectively, and then migrates to the placenta, fetal liver and spleen, followed by residing in bone marrow eventually for life-long hematopoiesis[15]. After birth, HSC primarily lives in bone marrow but mobilizes into circulation in response to injury.

HSCs are capable of self-renewing to replenish themselves while giving rise to all mature blood cells and immune cells[16]. Long-term HSCs (LT-HSCs) commonly maintain a quiescent state for protecting from detrimental stress, while enter into cell cycle upon hematological stresses, holding a marked capacity for self-renewal and long-term hematopoiesis[17]. On the other hand, short-term HSCs (ST-HSCs), which are derived from LT-HSCs, feature limited self-renewal potential and could differentiate into multipotent progenitors (MPPs). MPPs give rise to lineage-committed progenitors such as common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs)[1]. These lineage-committed progenitors subsequently give rise to specific lineage progenitors which produce all mature blood and immune cells. Hematopoietic progenitors have limited self-renewal capacity and differential potential and fail *in vitro* within several weeks[1].

$CD34^+$ cells are most enriched for HSCs, but only share 0.5%–5% of blood cells in human[16]. Actually, HSC represents a heterogeneous pool comprising of a variety of subpopulations which differ in functional and molecular phenotype[17]. In terms of molecular phenotype, different HSC subpopulation constitutively expresses a set of specific surface antigen markers. For instance, human HSCs are usually defined as $CD34^+CD38^-CD49f^+CD90^+CD45RA^-LIN^-$ cells, and $CD34^-CD150^+CD48^-CD117^+SCA1^+LIN^-$ is commonly used to define mouse HSCs[18]. On the other hand, different HSC subpopulation shows distinct self-renewal and multipotency capacity.

A single long-term HSC could reconstitute the whole blood system of lethally irradiated mice (with hematopoiesis failure)[19]. Self-renewing activity of these bona fide HSCs also enable reconstitution in secondary recipients for life-long hematopoiesis. Human HSCs capable of repopulating in NOD-SCID or NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice are termed NOD-SCID repopulating cells (SRCs), which are candidate HSCs[10]. In practice, activity of these long-term repopulating units is determined by competitive repopulating assay and serial transplantation assay,

while absolute number is quantified by colony-forming unit (CFU) assay and limiting dilution assay (LDA)[20].

2.2. HSC niche biology

HSC resides in unique bone marrow microenvironment (generally termed "niche") for maintenance and hematopoiesis[17,21,22]. Niche for HSCs could be divided into endosteal or vascular niche[23]. LT-HSCs commonly locate adjacent to endosteum while those active ST-HSCs reside closely to sinusoids[23].

Nowadays, by virtue of high-resolution imaging techniques and other advanced biotechnologies, spatial distribution of niche cell type and cell-to-cell interaction within niche could be visualized[24]. Endothelial cells and mesenchymal stromal cells (MSCs) maintain HSC *via* secretion of SCF (stem cell factor) and CXCL12[19,23]. Macrophages, osteoclasts and non-myelinating Schwann cells regulate HSCs through either direct or indirect effect[17,21]. Other cells, such as perivascular stromal cells and regulatory T cells, were also found to regulate HSCs[17,21]. Furthermore, sympathetic nerves are also known to regulate the HSC niche[21]. These niche cells and nerves work in concert to form a complex network to precisely regulate HSC cell fate decision through various extracellular cues such as cytokines and cell-to-cell interaction[17,21]. Furthermore, distinct niche exist for functionally and molecularly heterogeneous HSC pool[21].

Briefly, HSCs live in a perivascular niche, where their maintenance, self-renewal and differentiation are strictly regulated.

2.3. Signaling pathways orchestrating HSC fate decision

In addition to extracellular cues, intracellular factors, such as metabolism, key proteins and many signaling pathways, are also essential for regulating HSC fate decision[17]. Two conserved signaling pathways, Wnt and Notch signaling, which are widely present in various tissues and involved in many diseases, play important roles for regulating HSC fate decision[25−28]. Canonical Wnt pathway is mainly associated with self-renewal while non-canonical Wnt pathway regulates HSC quiescence[26]. Notch signaling plays a crucial role for HSC development during embryogenesis, and adult HSC differentiation toward T cells[27,28]. Apart from the two pathways, others such as TGF-β/Smad signaling are also involved in HSC fate regulation[29]. As many conserved pathways orchestrate to corporately form an intricate signal network for regulating HSC fate, the balance between HSC self-renewal and differentiation is highly ensured in a meticulous way. As a result, we will shortly describe basic transduction processes of Wnt and Notch pathways and discuss their involvement in HSC biology, and most importantly, in HSC expansion.

2.3.1. Wnt signaling

The Wnt signaling pathway is highly conserved during lengthy evolution throughout every stages and systems of all multicellular animals[30]. It plays an essential role in embryonic HSC development, adult HSCs self-renewal, lineages specification and maintenance[26,31−33]. A wide range of diseases (reviewed extensively by Nusse et al.[25] and Clevers et al.[34]) including hematologic diseases have been found to be associated with this pathway. The Wnt cascade represents 19 distinct Wnt ligands and more than 10 Frizzled receptors plus several co-receptors, which partially account for its broad spectrum of biological effects among many different species[25]. Broadly speaking, Wnt signaling pathway is divided into canonical and noncanonical pathway. Canonical Wnt pathway (Fig. 1), in which β-catenin is a critical mediator, modulates much on HSC self-renewal[26]. In the absence of Wnt signal, glycogen synthase kinase 3β (GSK-3β), CK1 (casein kinase 1) plus several tumor suppressor factors like Axin (axis inhibition protein) and APC (adenomatous polyposis coli) corporately form a destruction complex to phosphorylate β-catenin and lead to its proteasomal degradation upon ubiquitination[32]. Conversely, when Wnt signaling is on (in the presence of Wnt ligand), inactivation of destruction complex contributes to dissociation, buildup and accumulation of β-catenin, which promotes its translocation to the nucleus[32]. Dephosphorylated β-catenin binds to T cell factor (TCF) and form transcription activator complex for promoting expression of Wnt target genes[32]. Given the roles of Wnt cascade for HSCs development and cell fate decision at multiple stages of all metazoan species, the ancient pathway has been of great importance for regulating hematopoiesis.

In 2003, Willert and colleagues[35] demonstrated the active form of secreted Wnt3a protein enhanced mice BM HSCs self-renewal and led to subsequent *ex vivo* expansion. In consistence with this, loss of Wnt3a in BM niche compromised self-renewal capacity of LT-HSC[36]. Intriguingly, subsequent studies suggested that Wnt3a diminished long-term repopulating capacity by inducing human HSPC multilineage differentiation[37], though others indicated that constitutive activation of canonical β-catenin signaling blocks multilineage differentiation and compromises hematopoietic reconstitution. Until later compelling evidence explained these paradoxical results that it was the different level of Wnt signaling that accounted for distinct HSCs self-renewal capacities in the past studies[38]. High levels of canonical Wnt signaling promoted differentiation and diminished HSCs self-renewal capacity while mild Wnt activation enhanced HSCs self-renewal capacity and promoted expansion. Of note, Reya et al.[39] suggested a crosstalk between Wnt and Notch signaling that Wnt activation led to increased Notch1 expression, which revealed that both the two conserved signaling pathways worked cooperatively to regulate HSCs fate decision, especially self-renewal.

Early studies suggested addition of Wnt3a or Wnt5a proteins promoted HSC proliferation and self-renewal in culture[34,35]. Recently, Janda et al.[40] developed water-soluble surrogate Wnt agonists to activate canonical β-catenin signaling *in vivo* and displayed supportive effects in many organoid cultures *in vitro* comparably to Wnt3a, providing more benefits for studies on Wnt pathway and potential means for stem cells regeneration. Another Wnt protein Wnt9a was found to mediate early HSPC amplification[41]. All these evidence suggest an important role for Wnt signaling in HSC self-renewal and expansion, and it is possible to *ex vivo* expand HSCs through regulating Wnt pathway.

2.3.2. Notch signaling

As is the case with Wnt pathway, Notch signaling pathway is also highly conserved and occurs in numerous aspects of HSC biology, and its diverse biological effects depend on specific context and condition[28]. Notch pathway (Fig. 2) involves four receptors Notch1−4, five Notch ligands Delta (Delta1, Delta3, and Delta4) and Jagged (Jagged1 and Jagged2), target genes, and regulatory elements[27]. After Notch ligand binds to its receptor, the receptor undergoes two proteolytic cleavages, and then the Notch intracellular domain (ICD) is released, which recruits other components to form the transcriptional activation complex subsequent to translocation to the nucleus[27,28,42]. In cooperation with specific co-activators, the complex activates target genes transcription.

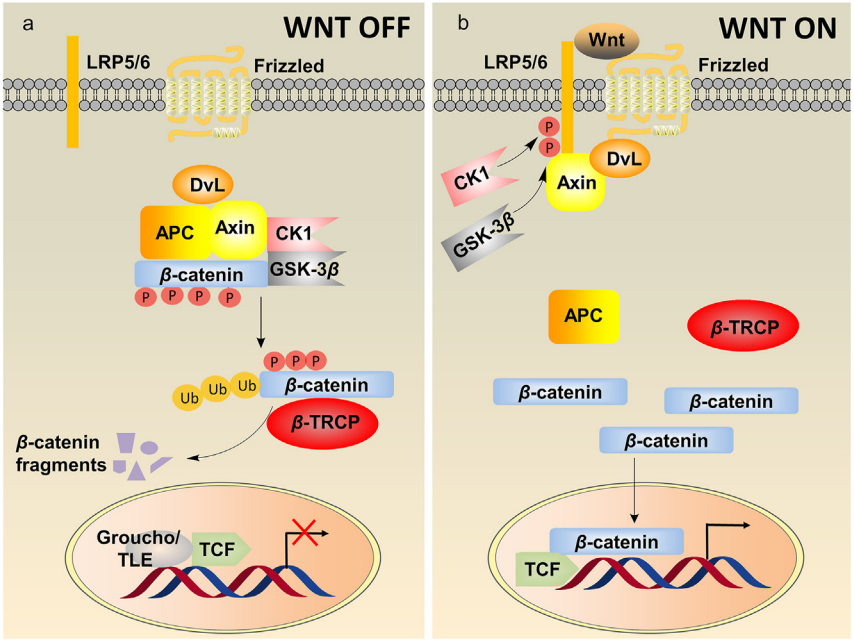

Figure 1 Canonical Wnt/β-catenin signaling pathway. (a) In the absence of Wnt protein, CK1 and GSK3β, as well as the anchor proteins Axin and APC form a destruction complex. β-Catenin in the cytoplasm is phosphorylated by CK1 and GSK3β, followed by bound to β-transducin-repeat-containing protein (β-TrCP), leading to ubiquitylation and degradation by the proteasome. In the nucleus, TCF recruits transcriptional co-repressors such as Groucho/TLE (transducin-like enhancer) proteins to block Wnt target genes transcription. (b) After Wnt binds to co-receptor LRP5/6 and Frizzled receptor, CK1 and GSK3β phosphorylate LRP5/6. β-Catenin is released from destruction complex and translocated to the nucleus, which binds to TCF to activate transcription.

Efforts in exploring effective protocols for *ex vivo* HSCs expansion employing Notch ligands have been made a lot despite the elusive functions of Notch signaling in adult HSCs. Jagged1, which is secreted by osteoblastic cells, occurred in a Notch1-dependent manner to support the expansion of HSCs both *in vitro* and *in vivo*[43]. Later, soluble Jagged1 was demonstrated to enable modest HSCs expansion *in vitro* and enhance long-term engraftment in NSG mice[44,45]. Negishi et al.[46] developed a transgenic hJ1-NOG mice model, in which BM niche was engineered to express human Jagged1 and transplanted human CB HSCs showed 5-fold expansion compared to control.

In 2003, Varnum-Finney et al.[47] found a combination of immobilized Delta1 and cytokines increased murine marrow precursor numbers by multi-log, and these expanded precursors were capable of short-term repopulation and long-term T-lymphoid reconstitution in mice. Subsequently, they further used this immobilized Delta1 to *ex vivo* expand human $CD34^{+}CD38^{-}$ cord blood progenitors, which exhibited a more than 100-fold increase in HSPC numbers, higher SRC frequency and enhanced short-term repopulating capacities in a low Delta1 dose culture[48–50]. These preclinical results proved preliminary safety and feasibility of Notch ligand for HSC expansion.

They then enrolled 10 patients to conduct a phase I clinical trial on Delta1-expanded CB unit (Table 1)[50]. 7 out of 10 patients remained alive without related diseases and established complete engraftment derived from donor. No infusional toxicities or other safety concerns were observed in all patients. A rapid neutrophil engraftment (median time 16 days) was observed compared to double CB unit's control. Intriguingly, hints from previous studies in which Delta1 contributes more to short-term repopulation and myeloid lineages reconstitution implied that the expanded unit is responsible for short-term repopulation, *i.e.*, short-term repopulating progenitor cells accounted for the rapid neutrophil engraftment. At six months after transplantation, donor hematopoiesis derived from Detla1-expanded unit is almost lost. Subsequent clinical observations indicated that the unmanipulated unit contributes mostly to long-term hematopoiesis while the expanded unit contributes to myeloid reconstitution[51]. Some researchers explained that immune cells present in the unmanipulated unit produce an immune rejection against the expanded unit, and the former becomes the "winning" unit[50–52]. Another rational explanation suggested that the portion of primitive HSC responsible for long-term repopulating depletes over the course of reconstitution[50,52]. Double armed clinical studies or studies on single expanded unit are still required to test whether this method expands bona fide HSC in cord blood unit and larger phase II/III studies are also required to evaluate whether rapid engraftment brings about improved overall survival of patients.

3. Cytokine and growth factor

Due to low dose of HSCs in human cord blood, people have starved for obtaining sufficient HSCs to satisfy the unmet need. Initial attempts to *ex vivo* HSC expansion were inspired by diverse cytokines and growth factors. SCF is the first defined cytokine for promoting HSC/HPC expansion *in vitro* despite low efficiency alone. Subsequently, several potent cytokines and growth factors were identified, including thrombopoietin (TPO), interleukin-3/6/11 (IL-3/6/11), Fms-like tyrosine kinase 3 ligand (FLT-3L), and G-CSF (granulocyte colony-stimulating factor)[53]. Although these cytokines alone are inefficient to support HSC expansion *in vitro*, a combination of several cytokines appears to be effective. Combination of SCF, FLT-3L, IL-3/6 and TPO are widely used for

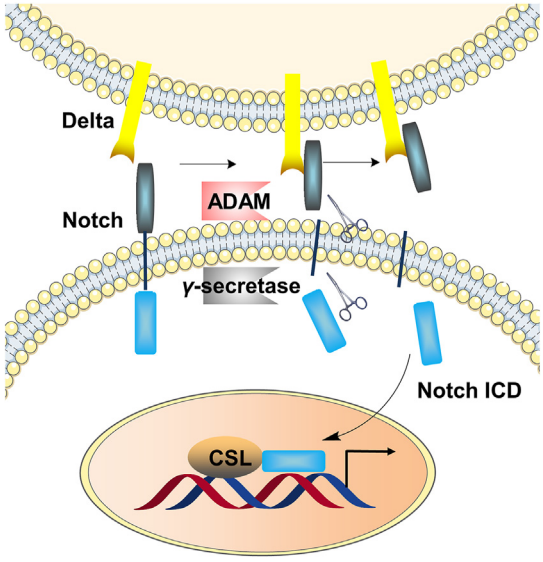

Figure 2 Overview of Notch signaling pathway. After Notch receptor and its ligand interact at the cell-to-cell surface, Notch receptor is cleaved twice by ADAM metalloprotease and γ-secretase. Notch ICD translocates into the nucleus, and then binds to the DNA binding protein CSL and recruits several co-activators to initiate Notch target genes transcription.

HSC *ex vivo* expansion in culture[53]. However, basic expansion culture systems are incapable of maintaining sustaining HSC expansion and eventually lead to depletion of HSC pool size.

Angiopoietin-like proteins (Angptls) family contains eight members, which play different roles in lipid metabolism, angiogenesis inflammation, cancer cell motility and HSC expansion[54]. Angptl3 was found to support HSCs maintenance, self-renewal and expansion in bone marrow niche[55]. Zhang et al.[56] suggested that Angptl2, 3, 5, and 7 induced expansions of long-term HSCs in synergy with SCF, TPO, FGF-1 and IGF-2, especially that Angptl2 and Angptl3 induce 24- and 30-fold net increase in LT-HSCs, respectively. Further studies by Akhter et al.[57] suggested that Angptl1, 2, 3, 4, 6 and 7 supported mouse HSCs survival while caused a 3- to 4.5-fold net expansion compared to cocktail of SCF, TPO, FGF-1 and IGF-2.

Sonic hedgehog (Shh) protein is one of three members of Hedgehog (Hh) family in human. Addition of Shh induced human $CD34^+CD38^-$ cells proliferation and doubled the CFU numbers after 7 days *in vitro* treatment[58]. Most importantly, Shh treated human primitive HSCs showed increased repopulating ability *via* inhibition of downstream BMP-4 expression[58].

Pleiotrophin (PTN) is a heparin-binding growth factor secreted by bone marrow perivascular stromal cells and vascular endothelial cells (ECs)[59]. Secretion of pleiotrophin from distinct niche compartments regulates HSC maintenance and self-renewal *via* inhibition of protein tyrosine phosphatase-ζ (PTPζ)[59–62]. PTN induced marked expansion of human CB HSCs and murine LT-HSCs in culture supplemented with other cytokines, and accelerated hematopoietic recovery of irradiated mice[62].

4. Small molecules for *ex vivo* HSC expansion

Although a variety of cytokines are capable of expanding HSCs, these factors are insufficient to maintain long-term and robust HSC expansion. Commonly-used cytokine cocktail (SCF, TPO, IL3/6/11, and FLT-3L) only provided transient (1–2 weeks) and limited HSC expansion. In a clinical trial, transient neutrophil recovery and lack of long-term engraftment *in vivo* was observed in Delta1-expanded CB graft. Thus, this strategy proved overall unsuccessful. Compared to cytokines or growth factors, small molecules have several advantages, such as definite effects, easily manipulated and low cost, which make them be widely investigated and used for *ex vivo* HSC expansion. Several small molecules proved safe and feasible in clinical trials. Recent years, many novel small molecules for HSC expansion have been reported. The development of small molecules is shown in Fig. 3. In this section, we will first review small molecules in clinical trials, and then outline novel small molecules according to their targets or properties. In order to clarify the medicinal chemistry involved in this field, binding mode and SAR studies of several molecules are also discussed in details. Small molecules in clinical trials, recent reported compounds, natural products and endogenous substances for *ex vivo* HSC expansion are summarized in Tables 1, 2, and 3, respectively.

4.1. *Small molecules in clinical trials*

4.1.1. *SR1*

A breakthrough occurred in 2010 that Boitano et al.[10] reported a promising purine derivative SR1 (**1**, Fig. 4) screened from a 100,000 compounds library to effectively expand HSC. In cooperation with cytokines combination, SR1 treatment led to a 50-fold expansion of CB $CD34^+$ cells, a 65-fold increase in CFUs and a 17-fold increase in SRC numbers. Mechanistically, SR1 binds to and directly inhibits aryl hydrocarbon receptor (AHR) in human and monkey, with a high inhibitory activity (IC_{50}) of 127 nmol/L, regardless of no activity on murine HSC. More significantly, a concentration of SR1 of 120 nmol/L resulted in a 50% increase in percent human CB $CD34^+$ cells compared to cytokines alone cultures.

A phase I/II clinical study (NCT01474681, Table 1) on SR1-expanded UCB unit combination with an unmanipulated unit have obtained encouraging outcomes including significant $CD34^+$ cells expansion, enhanced engraftment and rapid hematopoietic recovery[63]. In this study, SR1-treated UCB units underwent marked median expansion of $CD34^+$ cells at 330-fold over initial numbers. Compared to Notch-mediated expansion (Table 1) in which long-term engraftment in almost all settings was lastly derived from the unmanipulated UCB unit[50], SR1-expanded unit was responsible for durable and robust engraftment, similarly to that of nicotinamide[52,63]. In addition, a median time of 49 days to platelet recovery in SR1-group was much shorter than 89 days in control. Interestingly, 5 of 17 patients showed an exclusive mixed myeloid chimerism from both units and were observed with a particularly short median time at 7 days to neutrophil recovery, while this demonstrated no responsible for platelet recovery. However, high incidence of transplant-related mortality of 45%, and undesirable overall survival of 45% were observed. In addition, grade 3–4 acute GVHD remained a high percentage of 29%.

Recently, two phase II trials (NCT03674411 and NCT03406962) have enrolled participants to further evaluate SR1-expanded CB unit on hematologic malignancies or inherited metabolic diseases. It is encouraging that in 2018 FDA granted regenerative medicine advanced therapy (RMAT) designation to the SR1-expanded HSCs product (termed MGTA-456) for inherited metabolic disorders treatment.

Table 1 Detailed outcome of completed clinical trials involving manipulated UCB units.

Small molecule	ClinicalTrials.gov identifier	Status	Expansion effect[a]	GVHD incidence	Survival	Ref.
SR1	NCT01474681	Phase I/II completed	330-fold $CD34^+$ cells 854-fold TNCs	Grade III–IV aGVHD 29% (100 days)	OS 55% (12 months) TRM 45% (12 months)	63
UM171	NCT02668315	Phase I/II completed	35.4-fold $CD34^+$ cells	Grade II–IV aGVHD 64% (1 year) Grade III–IV aGVHD 10% (1 year) Chronic GVHD 17% (1 year) No moderate to severe chronic GVHD	TRM 5% (1 year) OS 90% PFS 74%	5
NAM	NCT01221857	Phase I completed	486-fold TNCs 72-fold $CD34^+$ cells	Grade II aGVHD 45% No grades III/IV aGVHD Chronic GVHD 18%	OS 82% (1 year) PFS 73% (1 year)	52
NAM	NCT01816230	Phase I/II completed	33-fold $CD34^+$ cells	Grade II–IV aGVHD 44% (100 days) Grade III–IV aGVHD 11% (100 days) Chronic GVHD 40% (2 years) Moderate to severe chronic GVHD 10% (2 years)	Nonrelapse mortality 24% (2 years) DFS 43% (2 years) OS 51% (2 years) Relapse 33% (2 years)	64
TEPA	–	Phase I/II completed	219-fold TNCs 6-fold $CD34^+$ cells	No grade III–IV aGVHD Grade II aGVHD 44% Chronic GVHD 50%	OS 90% (100 days) 70% (180 days) 40% (>21 months)	9
TEPA	NCT00469729	Phase II/III completed	400-fold TNCs 77-fold $CD34^+$ cells	Grade III–IV aGVHD 19.4% Grade II–IV aGVHD 31.6% Chronic GVHD 18.4%	OS 84.2% (100 days)	8
Notch-ligand	–	Phase I completed	562-fold TNCs 164-fold $CD34^+$ cells	Grade II aGVHD 90% Grade III aGVHD 10% Chronic limited GVHD 30%	OS 70% (354 days)	50
dm-PGE2	NCT00890500	Phase I completed	–	Grade I aGVHD 25% Skin-limited grade II aGVHD 16.7% Skin-limited cGVHD 16.7%	PFS 61.7% at 1 year 31.3% at 2 years OS 75% (1 year) 38.9% (2 years)	78

TNC, total nucleated cells; TRM, transplant-related mortality; OS, overall survival; PFS, progression-free survival; DFS, disease-free survival; aGVHD, acute GVHD; cGVHD, chronic GVHD.
[a]These data is relative to input cells content.

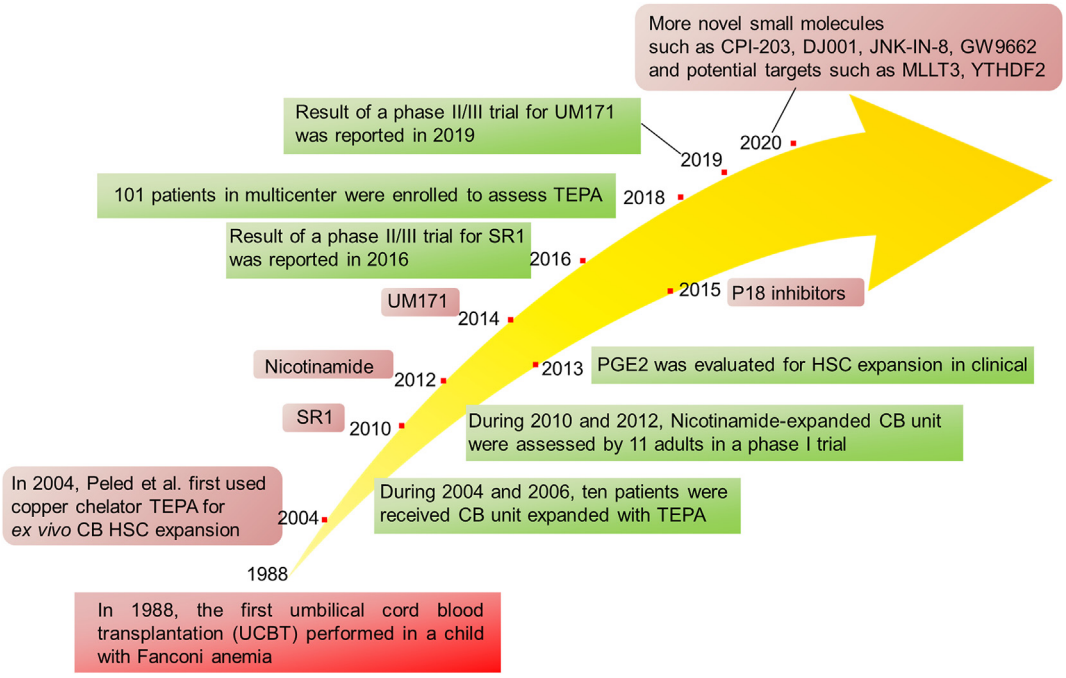

Figure 3 Chronological development of small molecules for *ex vivo* HSC expansion.

Of note, the author provided a SAR study on the C2, C6, and N9 position of purine ring of SR1 (Fig. 4). SAR studies suggested that 4-hydroxylphenthylamino moiety at C6 position is not essential and replacement with indole-3-ethylamino group results in 6-fold increase in expansion EC_{50} value of 0.02 μmol/L (**3**, Fig. 5). With respect to arylalkylamino group, the distance between nitrogen and aromatic ring is essential for *ex vivo* expansion activity; ethyl chain length is desirable and anilines results in loss of activity. Benzothiophene group is most preferred among a series of aryl/heteroaryl such as benzene, pyrimidine, furan and thiophene. Activity of these compounds could be modulated by replacement of *iso*-propyl with other aliphatic chain; 2-butyl outcompetes 3-butyl and *iso*-propyl, and it leads to a 4-fold increase in activity (EC_{50} 0.03 μmol/L) relative to *iso*-propyl, as exemplified by compound **2** (Fig. 5).

4.1.2. *UM171*

In 2014, Fares et al.[12] identified a potent small molecule for expanding $hCD34^+$ mPB cells, UM729 (**4**, Fig. 6), from 5280 compounds. After systematic structural optimization by SAR of

Table 2 Summary of recent small molecules for HSC expansion.

Compd.	Reported in	Target	Expansion effect	SRC expansion	Ref.
SW033291	2015	15-PGDH	–	2.4-fold	79
DJ001	2019	PTPσ	4.07-fold LSK	–	83
C7	2018	p38	3.7-fold $CD34^+38^-45^+45RA^-$	2.9-fold	87
JNK-IN-8	2019	JNK	8-fold $CD34^+38^-45RA^-90^+$	3.88-fold	91
005A	2015	p18	[a]ED_{50} 5.21 nmol/L	2.72-fold	98
CPI-203	2020	BET	5–10 folds HSCs	5–10-fold	99
GW9662	2018	PPAR-γ	7-fold $CD34^+CD38^-$	5-fold	101
Alexidine	2015	Ptpmt1	–	~2-fold	103
OAC1	2016	OCT4	2.2-fold $CD34^+CD38^-$ 2.8-fold HSCs	6.3-fold	106
NR101	2009	TPO	2.3-fold $CD34^+CD38^-$	2.3-fold	107
Eltrombopag	2018	TPO	–	1.7-fold	108
BIO	2011	GSK-3	2-fold CFU activity	–	111
CHIR99021	2005, 2012	GSK-3	7-fold TNC	5-fold	109,112
VPA	2005	HDAC	$CD34^+$ cell numbers↑	6-fold	121,122
LMK235	2018	HDAC5	–	4.3-fold	130
5azaD	2006	DNMT	2.5-fold $CD34^+$ 40-fold $CD34^+CD90^+$	–	132
zVADfmk/zLLYfmk	2010	Cysteine protease	3-fold $CD34^+CD38^-$	–	135
DEAB	2006	ALDH	11.6-fold[b] $CD34^+CD38^-$	3.4-fold	136

[a]Determined as the ratio of CFU-nmEM colonies.
[b]Relative to input, others without note are relative to control.

Table 3 Summary of natural products and endogenous substances for *ex vivo* HSC expansion.

Compd.	Target/signaling	Expansion effect	Ref.
Rapamycin	mTOR	–	112
Garcinol/isogarcinol	HATs	4.5-/7.4-fold HSCs 2.5-fold SRCs (garcinol)	118
TSA	HDAC	2.5-fold $CD34^+$ 40-fold $CD34^+CD90^+$	132
EchA	ROS inhibition PI3K/AKT activation	1.33-fold $CD34^+$ 1.7-fold CFUs	137
ASPP049	Hippo signaling	~1.5-fold $CD34^+CD38^-$ ~2-fold $hCD45^+$	143
Chrysin	p19, p57, MNDA, NR4A2, HOXB4	~1.5-fold HSCs 2.7-fold SRCs	145
Resveratol	–	1.84-fold $CD34^+/CD133^+$	138–140
CAPE	HIF-1α, SDF-1α and VEGF-A	1.47-fold CFUs 1.77-fold SRCs	141,142
5-HT	Apoptosis inhibition	1.73-fold CFU-F 12.2-fold $CD34^+$	146
α-Tocopherol	ERK1/2	2-fold LSKs	147
ATRA	RA receptor	4-fold CFUs	148,149

more than 300 analogs, UM171 (**5**, Fig. 6) was confirmed as the top candidate possessing a strong expanding activity of 17–19 nmol/L which was over 10-fold than that of UM729[12]. Both UM171 and UM729 could enhance repopulating capacity of macaque $CD34^+$ cells by three folds compared to control. The numbers of LT-HSCs in fed-batch culture (established medium delivery system) supplemented with UM171 increased by over 13-fold. Furthermore, transplantation assays showed that UM171 maintained long-term repopulating capacity of LT-HSCs and therefore enabled persistent engraftment in secondary recipients for over 18 weeks. Mechanism studies suggested that UM171 did not inhibit AHR while repressed gene expression involved in erythroid and megakaryocytic differentiation, and highly up-regulated TMEM183A expression as well as transcripts of some surface molecules.

A phase I/II trial (NCT02668315, Table 1) was conducted between Feb 2016 and Nov 2018 aimed to evaluate safety and feasibility of single UM171-expanded CB unit on 27 patients suffered from hematologic malignancies[5]. In the phase II cohort, 22 patients received single expanded CB unit despite slow neutrophil engraftment at a median time of 18 days relative to that of nicotinamide[64] and SR1[63]. UM171 outcompeted both two compounds with respect to low incidence of grade 3–4 acute GVHD (10%) and no moderate to severe chronic GVHD as well as low transplant-related mortality (5%, in 1 year). Furthermore, overall survival and progression-free survival was remarkable, 90% and 74%, respectively. Taken together, UM171 expanded single CB unit appears to be safe and feasible because of low transplant-related mortality and low incidence of GVHD in addition to no graft failure. A phase III trial is needed to further compare UM171 and standard condition (two unmanipulated CB units).

In addition, they also initiated two phase II clinical trials to further evaluate this HSC product in terms of transplant-related mortality and relapse-free survival on leukemia or myelodysplasia patients (NCT03913026 and NCT04103879). Furthermore, a

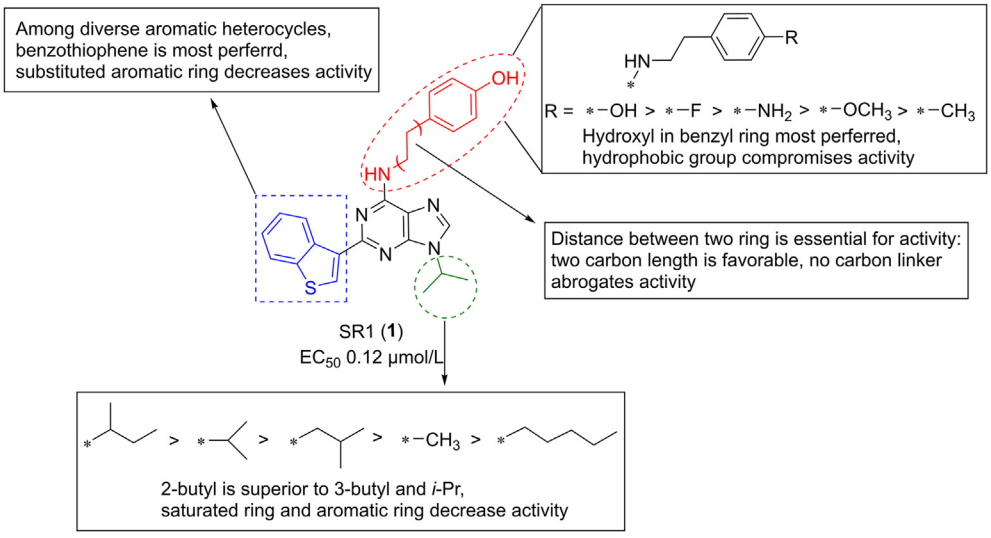

Figure 4 SAR studies of SR1.

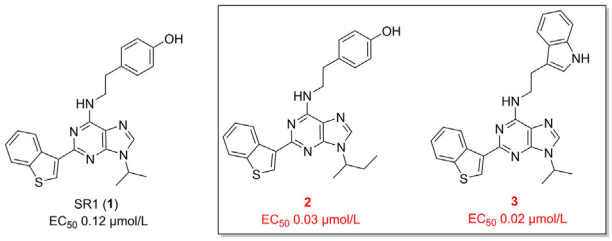

Figure 5 Structures and activities of compounds **1–3**.

clinical study (NCT03441958) has been set up to evaluate the safety and efficacy on multiple myeloma.

Recently, Feng et al.[65] designed and synthesized two series of pyrimidoindole analogs in which the tetrazol group substitutes in C6 or C7 to discuss the SAR of UM171 (Fig. 6). These analogs were tested to increase the percentage of $CD34^+CD38^-$ cells which are enriched for HSCs. They focused on assessing the effect of tetrazol group at C6 or C7 position and effect of substituents at C4 position on HSC activity. SAR studies revealed that substitution site of tetrazol group affected activity profoundly (Fig. 6). Same substitution at C6 or C7 position (**6** and **7**) shows completely distinct activity. **6** shows an EC_{50} value of 473.43 nmol/L while **7** has no activity. Although both **8** and **9** bear a same tetrahydroisoquinolino ethylamino and share a same EC_{50} value of 30 nmol/L, the different site of tetrazol group results in about three-fold difference in expanding $CD34^+CD38^-$ cells. The multilineage potential of **8**-treated HSC was maintained with a 1.4-fold increase in CFUs. **8** is a promising agent, but further studies are needed to evaluate whether **8**-expanded HSCs maintain long-term repopulating capacity in animal model.

4.1.3. Nicotinamide

As a form of vitamin B-3, nicotinamide (NAM, **10**, Fig. 7) is essential for human living and have diverse biological effects. Peled et al.[11] observed its unique effect on HSCs expansion. NAM treatment resulted in an 80-fold increase in CB $CD34^+$ cells, along with a robust expansion of $CD34^+CD38^-$ subpopulation. Transplantation assays suggested several folds increase in NOD/SCID repopulating cells after NAM treatment and improvement of engraftment potential of NAM-treated HSCs. NAM appeared to attenuate differentiation and enhance self-renewal capacity. Furthermore, NAM improved homing efficiency of $CD34^+$ cells dependent on CXCR4 to SDF-1 (stromal cell derived factor-1, also known as chemokine CXCL12, regulator for HSC homing) by 6 folds. Mechanistically, NAM selectively inhibited SIRT1 (Sirtuin1, class III NAD-dependent histone deacetylase) to exert the expansion effect. A specific SIRT1 inhibitor EX-527 (**11**, Fig. 7) also has similar expansion effect with NAM[11].

As inspired by remarked preclinical outcomes, there has been a class of clinical studies going on. Gamida Cell first conducted a phase I trial (NCT01221857, Table 1) which enrolled 12 patients in Duke University School of Medicine during Dec 2010 and Aug 2012 to preliminarily assess the engraftment of NAM-expanded CB HSC product, NiCord[52]. This novel protocol provided long-term engraftment in 8 of 10 patients for more than 2 years. Not only NAM-treated CB units achieved rapid hematopoietic recovery that median time to neutrophil and platelet engraftment was at 13 and 33 days compared to 25 and 37 days in historical control, respectively, but also showed high overall survival of 82% and progression-free survival of 73%. Among eight patients with partial or complete myeloid chimerism derived from NAM-expanded unit, six of which achieved stable NAM-derived T-cell engraftment. Coinfusion of an uncultured T cell fraction could possibly account for the predominance of the NAM-expanded unit. Also of note, there were no cases of acute grade 3−4 GVHD but two developed chronic GVHD.

Once the safety and efficacy of NAM were justified (NiCord-provided hematopoiesis has sustained for more than 7 years), they further dissected whether single CB unit expanded with NAM could achieve successful engraftment (NCT01816230, Table 1)[64]. As a result, 36 patients were enrolled to receive single expanded unit at 11 places. In this cohort, hematopoietic recovery appeared to be fast with median time to neutrophil at 13 days (21 days for control, 146 patients), accounting for less hospital stay and decreased incidence of infection. Most significantly, this NAM-

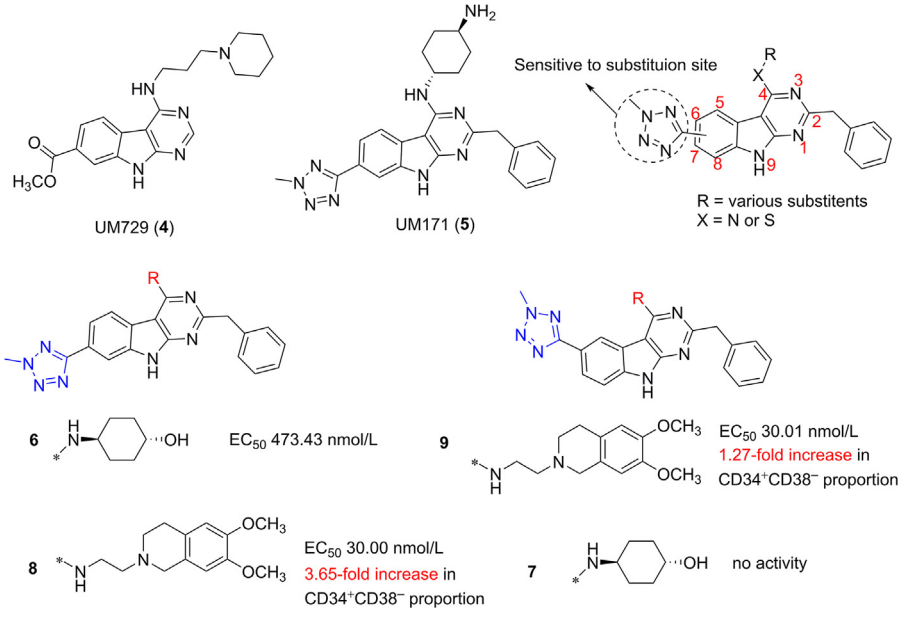

Figure 6 Discovery and structural optimization of UM171.

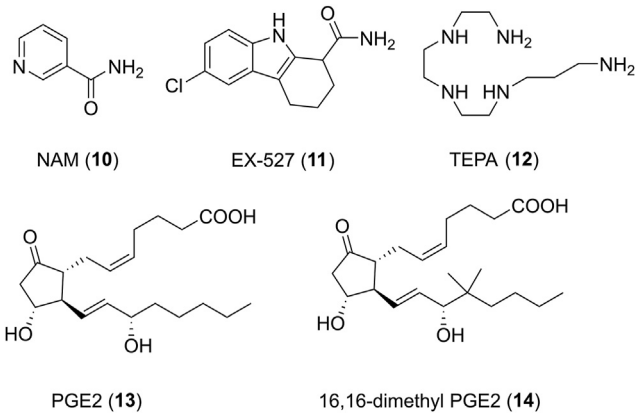

Figure 7 Structures of compounds **10**−**14**.

based therapy demonstrated low incidences of severe acute or chronic GVHD, and high disease-free survival of 43% and low nonrelapse mortality of 24% despite possibly higher risk of relapse in 2 years (Table 1).

Previously, NiCord have been received orphan drug designation for AML treatment by European Medicines Agency's (EMA's) Committee and for treatment of several hematologic malignancies by FDA. Furthermore, FDA granted breakthrough therapy designation for NiCord in blood cancer. Recently, EMA have extended orphan drug designation for NiCord to treatment for hematopoietic stem cell transplantation. In the future, a phase III trial (NCT02730299) for NiCord will be conducted on 120 patients with hematologic malignancies on multicenter.

4.1.4. TEPA

Early studies have indicated that cord blood-derived HSC differentiation was inhibited and proliferation was enhanced in the context of low copper condition by addition of copper chelator, tetraethylenepentamine (TEPA, **12**, Fig. 7) while differentiation was accelerated under high copper condition[66,67]. TEPA resulted in a stable and robust increase in CB-derived $CD34^+$ cells and its primitive progenitor subpopulation, which showed enhanced repopulating capacity in NOD/SCID mice[68,69].

In 2008, Gamida Cell reported a phase I/II trial (Table 1) to evaluate StemEx (an expanded $CD133^+$ fraction from single CB unit along with the unmanipulated fraction) transplantation on 10 patients with hematologic malignancies[9]. Despite a limited $CD34^+$ cell expansion (6-fold average) and low TNCs (total nucleated cells) content after TEPA expansion, high overall survival of 90% in 100 days was seen in nine patients and no cases of grades 3−4 acute GVHD occurred. However, slow neutrophil and platelet recovery (median time 30 and 48 days, respectively) was observed.

Base on the feasibility of StemEx, 101 patients were enrolled at 25 sites, and 295 patients from international registries received double unmanipulated CB units transplantation as a comparator (NCT00469729, Table 1)[8]. Regarding hematopoietic recovery, median time to neutrophil and platelet of TEPA group was 21 and 54 days, comparing 28 and 108 days in control, respectively. Although the 100-day overall survival of the TEPA group (84.2%) is superior to that of control group (74.6%), the 180-day overall survival is similar. The primary cause for the ascending late infection and mortality is reduced immune cells infusion. Also, the incidence of acute and chronic GVHD is similar between two groups.

4.1.5. PGE2

Early in 40 years ago, accumulated evidence revealed PGE2 (**13**, Fig. 7) has a stimulative effect on HSCs and mature blood cells despite many contradictory observations[70]. Until 2007, a stable PGE2 derivative 16,16-dimethyl-PGE2 (dmPGE2, **14**, Fig. 7) was found by North et al.[71] to increase CFUs and SRC frequency in murine which was transplanted with dmPGE2-treated BM cells. Hoggatt et al.[72] reported that short *ex vivo* exposure of murine HSCs to dmPGE2 promotes their homing, survival and proliferation, with an enhanced long-term repopulating capacity.

Furthermore, dmPGE2 accelerated hematopoietic recovery and had hematopoiesis-protective effects in sublethally irradiated murine and zebrafish[73,74]. PGE2 could activate Wnt signaling pathway to promote HSC self-renewal[75]. HSCs express four G-protein coupled E-prostanoid receptors, EP1−4, each of which distinguishes in mode of actions and biological outcomes[76]. In addition, the PGE2/Wnt interaction is conserved in vertebrate for regeneration and recovery, and binding of PGE2 to EP4 regulates the stability of β-catenin *via* cAMP/PKA signaling and subsequently activates Wnt pathway[74−76].

Preclinical studies in which dmPGE2-expanded UCB unit enhanced engraftment in NOD/SCID mice, showed safety and potency of dmPGE2 for hUCB transplantation[77]. All these evidence provided safety, efficacy and definite molecule mechanisms of dmPGE2, and paved the way to phase I clinical trial (NCT00890500, Table 1). A rapid neutrophil recovery (median time 17.5 *vs.* 21 days in control) and preferential long-term engraftment (10 of 12 treated patients) was observed in patients who were infused a dmPGE2-UCB unit along with an unmanipulated CB unit compared to double unmanipulated UCB units[78].

4.2. Recent advances of novel small molecules

4.2.1. 15-PGDH inhibitor

As is previously described, PGE2 promotes HSC homing and expansion and enhances hematopoiesis after irradiation[71,77]. A clinical trial has proved that PGE2 or dmPGE2 enhanced CB units engraftment in patients[78]. Inhibiting prostaglandin-degrading enzyme 15-PGDH enhances PGE2 levels *in vivo*, and favors tissue repair and hematopoietic recovery[79,80]. Either *15-PGDH* knockout or pharmacologic inhibition of 15-PGDH resulted in increase in numbers of HSC and mature blood cell[79]. As a result, 15-PGDH inhibitors are promising agents for *ex vivo* HSC expansion. Zhang et al.[79] screened from 230,000 compounds and identified a potent 15-PGDH inhibitor SW033291 (**15**, Fig. 8) with a dissociation constant $K_i = 0.1$ nmol/L and inducing 2-fold increase in PGE2 levels *in vivo*. Bone marrow cells *ex vivo* treated with **15** were significantly enhanced with repopulating capacity. Also, **15** benefited irradiated mice hematopoietic recovery after allogeneic transplantation. Due to poor solubility of **15**, they did further medicinal chemistry work to improve its druglike properties[81]. Through a systematic SAR studies, they obtained four sulfoxides with better potency profile both *in vitro* and in cells but also improved solubility. Hydrochloride salt of (+)-**16** (*R*-enantiomer of **16**), was proved over 10,000-fold more soluble than racemic **15** (Fig. 8).

SAR studies suggested that sulfoxide moiety is essential for enzyme inhibition activity and cellular activity (Fig. 9). Conversion of sulfoxide to sulfide or sulfone led to loss of substantial activity and 10-fold less active than **15**, respectively. In addition, replacement of sulfoxide with carbonyls attenuated potency. Putative inhibitor-enzyme binding model suggested that sulfoxide

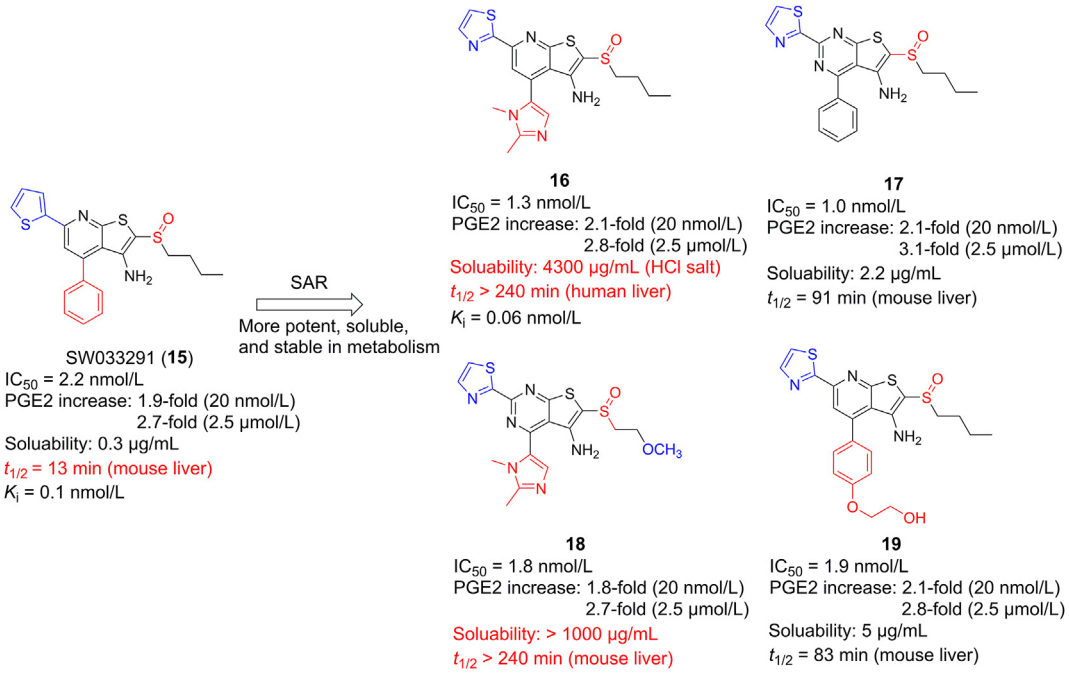

Figure 8 Structures, biological activities, solubility, and metabolism profile of compounds **15–19**.

mimics the charge distribution and the transition state of 15-hydroxyl of PGE2, and therefore blocks key Tyr151 of 15-PGDH for degrading PGE2. This possibly explained *R*-enantiomer of sulfoxides significantly outperformed *S*-enantiomer in terms of *in vitro* enzyme inhibition and metabolism stability *in vivo* as exemplified below by (+)-**16**, (+)-**17** and (+)-**19**. Size and properties of sulfoxide side chain dramatically affected inhibition activity and activity in cells. Introducing ether group to alkyl side chain retained activity while improved 10-fold solubility. The phenyl group in pyridine ring was dispensable for enzyme inhibition and allowed for replacement with heterocyclic rings, but important for maintaining activity in cells. Introducing dimethylimidazolyl to replace phenyl moiety yielded **16** whose hydrochloride salt is 10,000-fold more soluble in water than **16**. *S*-Enantiomer of **16** almost lost activity both *in vitro* and *in vivo*. (+)-**16** has marked metabolism dynamics profile in human liver with a desirable half-time of over 240 min without any toxicity. Substitution on phenyl ring maintained activity and changed molecular polarity with an improvement of solubility, such as **19** which features an ethylene glycol moiety and led to 10-fold solubility improvement and longer half-time in mouse liver while as active as **15**. Thienyl group in **15** is not essential and is of well

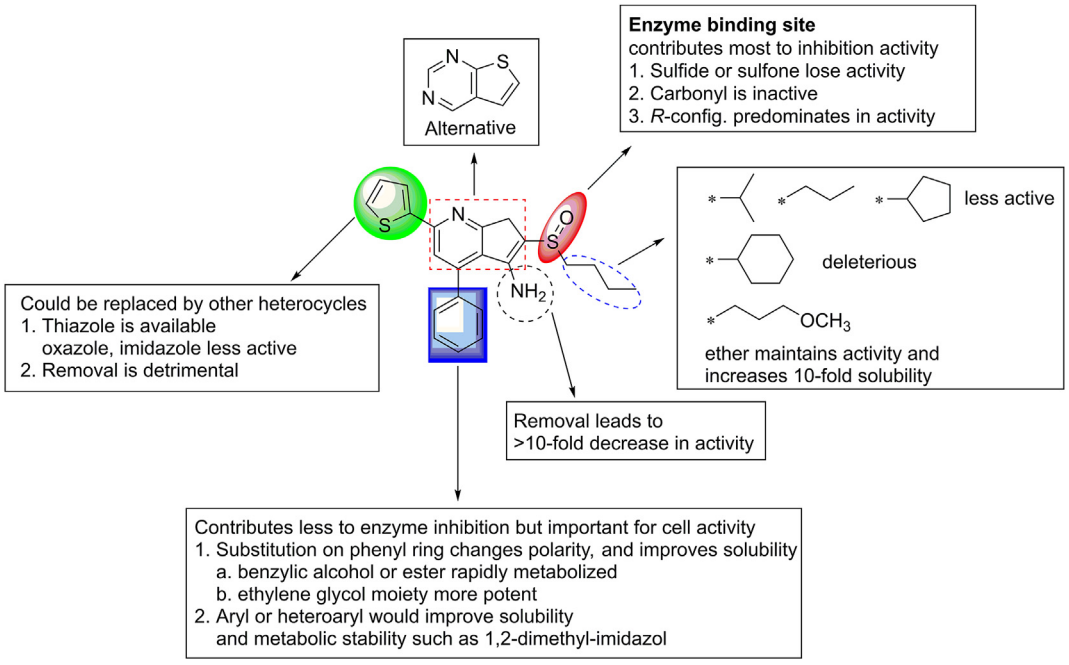

Figure 9 SAR summary of 15-PGDH inhibitors.

tolerance for heteroaryls, such as thiazolyl and oxazolyl, despite slightly decreased activities. Furthermore, in addition to thienopyridine, thienopyrimidine as central ring also maintained activity, such as **17** or **18** (Fig. 8). All four optimized sulfoxides doubled PGE2 levels in bone marrow of mouse at 3 h and were more metabolically stable.

(+)-**16** possesses 10,000-fold solubility and better *in vivo* activity relative to **15** by replacement of phenyl with a 1,3-dimethylimidazolyl and substitution of thiophene with thiazole. In consistence with **15**, (+)-**16** was demonstrated to enhance HSC homing and engraftment, and accelerated hematopoietic recovery in animal HSCT model, without any adverse effects[80]. Thus, 15-PGDH inhibitors may also serve as therapeutic agents for ameliorating engraftment after HSCT.

4.2.2. *PTPσ inhibitor*

Protein tyrosine phosphatase (PTP) family comprises of several therapeutic members, whereas progresses in druggable small molecules targeting on PTPs are lagging behind. In 2015, Quarmyne et al.[82] found that PTPσ was expressed by murine and human HSC and loss of PTPσ resulted in enhanced long-term repopulating capacity. HSCs derived from *Ptprs* (encode PTPσ) -knockout mice were capable of long-term repopulating, and negative selection of receptor PTPσ ($PTP\sigma^{-}$) CB cells comparably resulted in increased repopulating capacity comparing with those of $PTP\sigma^{+}$. Recently, Zhang et al.[83] screened from 80,000 compounds according to similar structure features of a known PTPσ inhibitor with IC_{50} of 2.3 μmol/L (Fig. 10). Two 2-arylamino-1-arylpropenone inhibitors were identified, one of which compound DJ001 (**20**) showed an IC_{50} of 1.5 μmol/L and had strong *ex vivo* HSC expansion activity. They further synthesized more than 50 analogs to provide **22**, while **22** is less active than **20** (Fig. 10). **20** suppressed HSCs apoptosis, enhanced HSCs survival and proliferation, and promoted hematologic recovery in irradiated mice[83]. It is worth noting that **20** promoted irradiated human HSCs self-renewal and hematopoietic reconstitution. Mechanistic studies discovered that **20** improves hematopoietic recovery *via* activation of RAC1 and downstream signaling cascade activation (*e.g.*, BCL-X_L, CDK2, and ERK1/2) in response to selective and allosteric binding to PTPσ.

4.2.3. *Small molecule regulators of MAPK signaling*

Simply speaking, three primary classes of kinase ERKs, JNKs and p38MAPKs constitute mitogen activated protein kinases (MAPKs) family[84]. For HSC expansion, there have been many studies focusing on small molecules targeting MAPKs signaling, and great achievement has been achieved. All these studies suggested that targeting MAPKs may be a promising strategy for promoting HSC expansion.

4.2.3.1. p38 inhibitor. p38 signaling is involved in erythroid development and negatively regulates myeloid differentiation in normal conditions. Activation of p38 compromises HSC self-renewal capacity and affects quiescence state maintenance. Pharmacologic inhibition of p38 could enhance multilineage hematopoiesis recovery in myelodysplastic syndromes (MDSs)[85]. Inhibition of p38 by SB203580 (**23**) resulted in both hUCB $CD133^{+}$ HSPCs and mouse $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK, enriched for HSCs) cells expansion and enhanced self-renewal and repopulating capacity (Fig. 11)[86]. Recently, Bari et al.[87] established ~50 azole molecules by SAR studies based on **23**. Replacement of 4-methylsulfinylphenyl by 1-fluoronaphthalen-2-yl at C3 of core imidazole ring of **23** provided compound **C7** (**24**, Fig. 11), which shows an over 1500-fold expansion of HSPC of unfractionated CB-MNC (mononucleated cell), 2.9-fold higher than that of **23**. An over 1.7-fold HSPC expansion occurred in **24**-treatment group relative to **23** despite similar repopulating capacity. Interestingly, **23** in combination with UM171 robustly promoted $CD34^{+}$ HSPC expansion with at least 6-fold higher than **24** alone.

SAR studies suggested that replacement of 4-fluorophenyl on imidazole ring with 3-(methyl)phenyl or 3-(trifluoromethyl)phenyl decreased activity, as exemplified by **25** (Fig. 11). In terms of

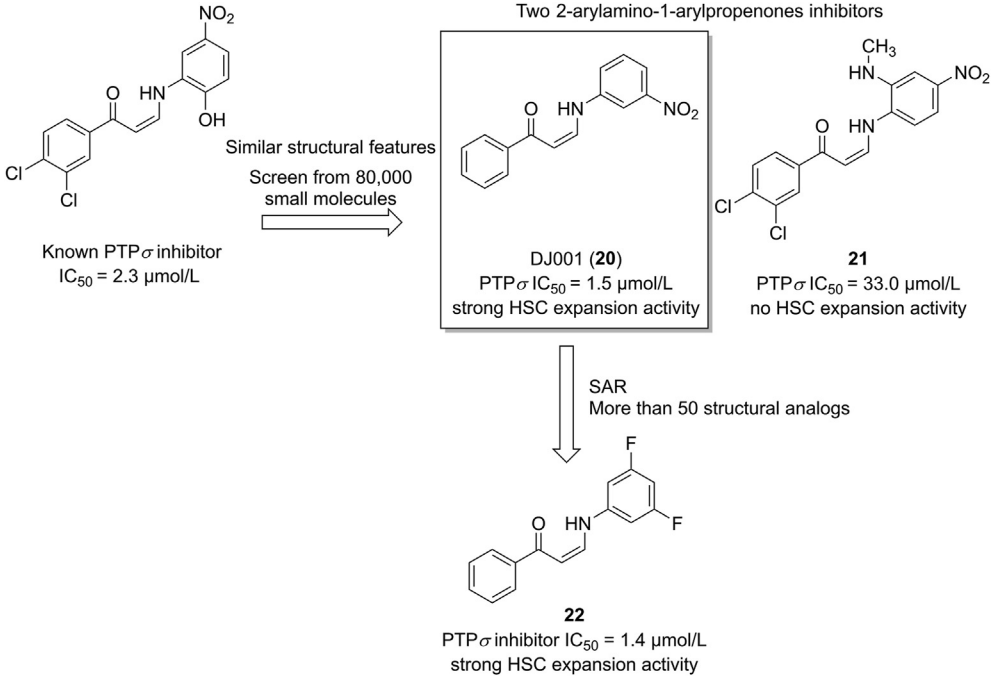

Figure 10 Discovery of PTPσ inhibitor DJ001.

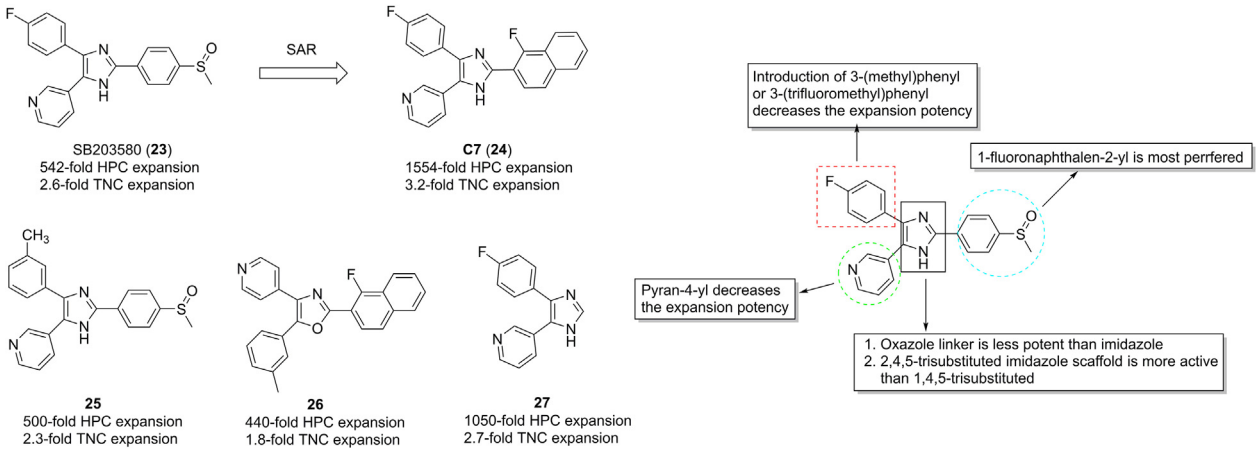

Figure 11 Structures of **23–27** and SAR summary of 2,4,5-trisubstituted imidazole p38 inhibitors.

imidazole core, oxazole almost lost activity, such as **26**. In addition, 2,4,5-trisubstituted imidazole was better than 1,4,5-trisubstituted imidazole. At C5 of imidazole ring, pyran-4-yl was less active than pridine-3-yl. Conversion of sulfoxide phenyl with 1-fluoronaphthalen-2-yl provided **24**, which is significant more active than **23**. Removal of phenyl methyl sulfoxide yielded **27**, which was about 2-fold more active in HSC expansion than **23**.

4.2.3.2. JNK inhibitor. Of the mitogen activated protein kinases (MAPKs) family, the c-Jun N-terminal kinases (JNKs) consist of several exclusive members JNK1/2/3[84]. JNK signaling over-activation is commonly present in cancer, inflammation and neurodegenerative diseases, and JNK pathway plays an important role for the normal hematopoiesis and development of leukemia[88,89]. Recently, investigators from Peking University used a potent JNKs inhibitor JNK-IN-8 (**28**, Fig. 15) to effectively expand human CB HSPC and its primitive subpopulations[90,91]. **28**-treated HSCs resulted in a remarkable 3.88-fold increase in SRC frequency in primary recipients and could reconstitute secondary recipients for over 21 weeks. Early in 2011, Zhang et al.[90] demonstrated that **28** is a potent selective JNKs covalent inhibitor. Among several families of kinase, **28** specifically inhibited JNKs of CMCG family. The introduction of flag methyl in **28** increased substantially selectivity over JNKs relative to its analog without methyl whose co-crystal structure with JNK3 (PDB 3V6S) suggested that acrylamide warhead (reactive groups for covalently targeting conserved residues) was covalently bound to the conserved Cys154 and the aminopyrimidine motif formed hydrogen bonds with the kinase hinge residue Met149. This "methyl effect" has been early observed with imatinib[92].

4.2.4. p18 inhibitor

As a member of INK4 CKI (cyclin-dependent-kinase inhibitor) family, p18 is responsible for regulating cell cycle *via* inhibition of downstream CDK4/6 signaling. Many studies have demonstrated deletion of p18 resulted in enhanced self-renewal capacity and robust *ex vivo* expansion of HSC without depletion in a long period[93–96]. Others also indicated a special role for p18 in preserving HSC quiescence status[97]. Therefore, Gao et al.[95] performed an *in silico* virtual screening for p18 inhibitors based on computational predication of binding pocket of p18/CDK6 complex crystal structure (PDB 1G3N) and further confirmed by *in vitro* and *in vivo* assays (Fig. 12). Compound P18IN011 and

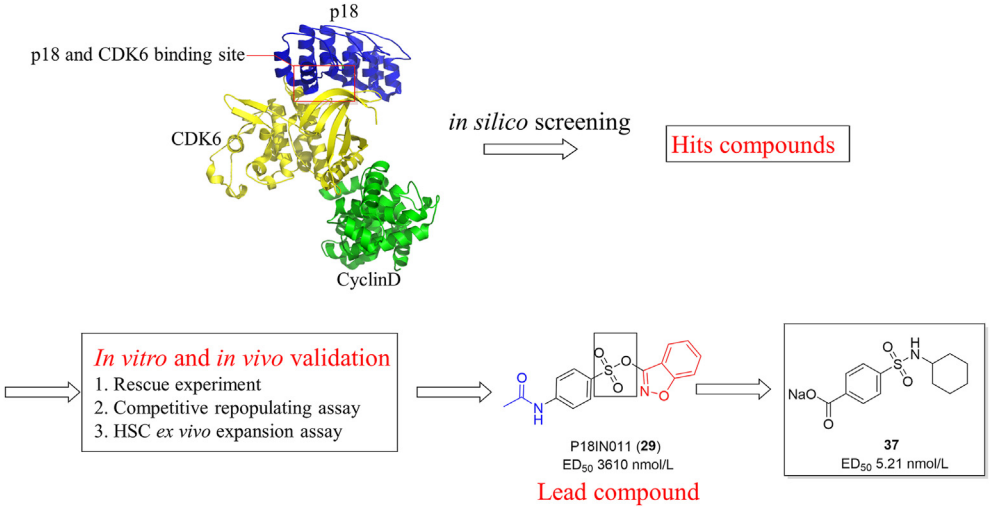

Figure 12 Identification and optimization of the first specific p18 inhibitor for HSC expansion.

P18IN003 (**29** and **37**, Fig. 13) were identified as hit compounds from library. Strong hydrogen bonds and hydrophobic interactions were observed between **29** and specific residues in interface of binding site of p18 and CDK6 by docking studies, suggesting **29** likely blocks or interferes the inhibition of p18 towards CDK6. Both **29** and **37** could support *ex vivo* expansion of functional mouse HSCs with ED_{50} of 3.61 and 6.12 μmol/L, respectively, and enhanced engraftment capacity of treated-HSC in primary and secondary recipients. They also found that these p18 inhibitors did not influence the cell cycle status and apoptosis but promoted self-renewal division.

Based on previous identification of p18 as lead compounds, they further used medicinal chemistry methodology to obtain optimized p18 inhibitors through a virtual screening, SAR and validation process[98]. Structural modification yielded three series compounds with four representative compounds possessing ED_{50} values less than about 10 nmol/L. SAR studies suggested that benzothiazine moiety is not the key group and lactone ring appears more potent especially bearing *iso*-propyl group (**30–32**, Fig. 13). Sulfonyl is essential for H-bonding interaction and phenyl provides suitable distance between adjacent groups, which is important for extensive H-bonding interaction and hydrophobic interactions in conserved pocket of p18/CDK6. Also, replacement of acetamide group by other groups such as methyl group (**33**, Fig. 13) brings about 5-fold increase in activity relative to **31**, indicating acetamide group is not crucial. Although benzoic acid **34** exhibits similar activity with **31**, its sodium salt **35** shows strongest HSC expansion activity with an ED_{50} value of 5.21 nmol/L, which is over 690-fold active than **29** and has better solubility properties. Interestingly, activity of these compounds is very sensitive to configuration when replacement of benzothiazine ring with amino acid such as tyrosine (**36**, Fig. 13) showed that *R*-enantiomer outcompeted *S*-

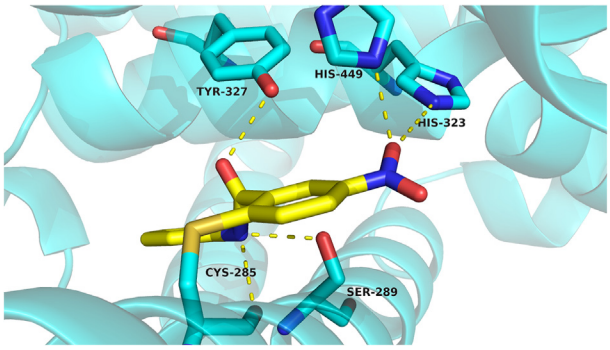

Figure 14 X-ray cocrystal structure of compound **39** covalently bound to the PPAR-γ ligand binding domain (PDB 3B0R). The compound (yellow sticks) is covalently attached to Cys285 of PPAR-γ protein in a S_NAr manner. Extensive hydrogen bonds (yellow dashed line, distance < 3.5 Å) are observed between the compound and several residues (cyan sticks).

over 1000-fold. Compound **35** could robustly *ex vivo* expand human CB HSC and enhance about three times repopulating capacity. Furthermore, these expanded LT-HSCs enabled stable engraftment in SCID mice for at least 4 months without any cytotoxicity and leukemia potential[96,98].

4.2.5. *BET inhibitor*

Bromodomain-containing proteins (BCPs) are able to regulate transcriptional process *via* recognition of epigenetic modification. Recently, Hua et al.[99] screened 10 small molecules targeting BCPs, with a specific bromodomain and extra-terminal motif (BET) domain inhibitor CPI-203 (**38**, Fig. 15) being identified to

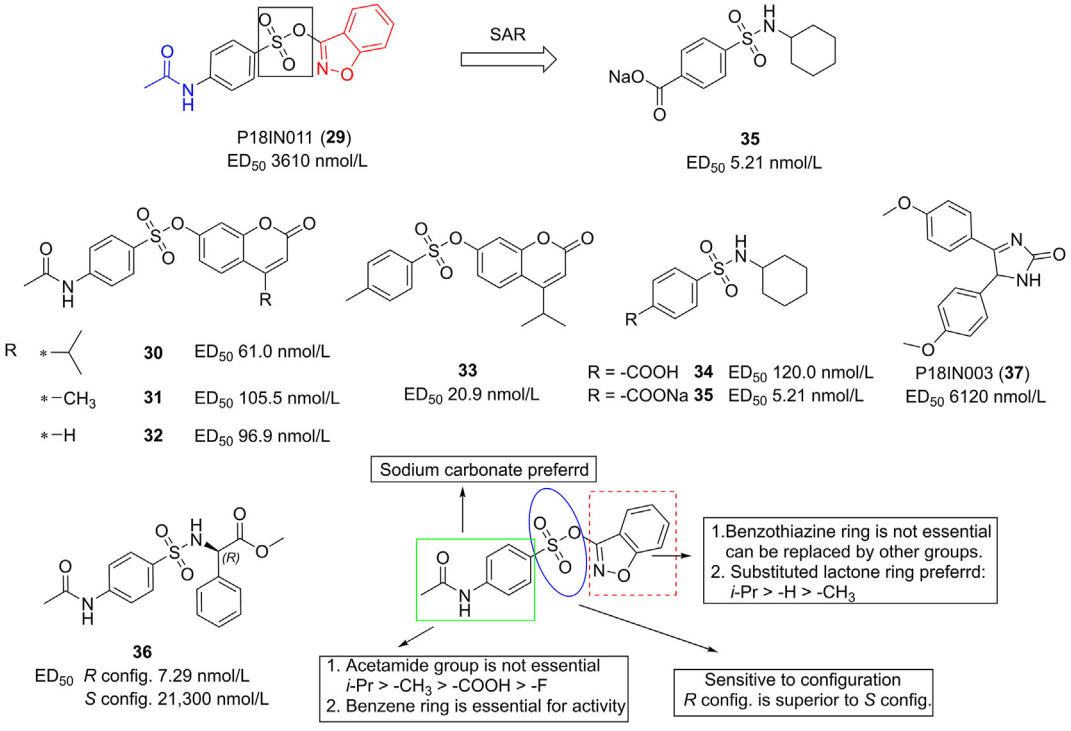

Figure 13 Structures and activities of **29–37**, and SAR summary of p18 inhibitors.

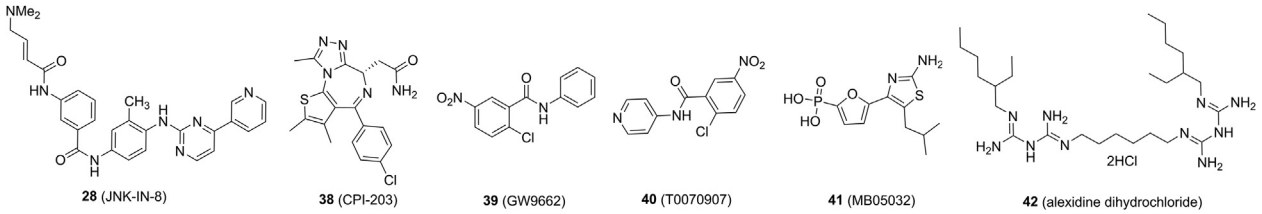

Figure 15 Structures of **28** and **38–42**.

expand human CB HSC and promote long-term repopulating capacity *in vivo*. It is interesting that CPI-203 also promotes megakaryocyte expansion, as previously reported.

4.2.6. *Small molecule regulators of energy metabolism*

As a member of nuclear hormone receptors, peroxisome proliferator-activated receptor γ (PPAR-γ) is an active regulator in glycometabolism and is involved in many cancers and metabolic diseases[100]. Recently, a study by Guo and co-workers[101] revealed that PPAR-γ inhibition, by its antagonist GW9662 (**39**, Fig. 15), resulted in robust *ex vivo* expansion in CB HSPCs. These expanded HSPCs could repopulate primary and secondary NSG mice recipients and exhibited a 5-fold higher frequency of SRC relative to control. In addition, FBP-1 expression was also decreased in CB HSCs in the presence of **39**, along with a host of differentiation-related genes. Also, glycolysis activity was enhanced following **39** treatment and expansion effect was abrogated by removal of glucose. These results suggested elevated glycolysis level *via* PPAR-γ−FBP-1 axis promoted HSC expansion and inhibited differentiation. Of note, another potent PPAR-γ antagonist T0070907 (**40**) and a specific FBP-1 inhibitor MB05032 (**41**) also promoted *ex vivo* expansion of CB HSC (Fig. 15)[101].

An X-ray cocrystal structure of **39** in complex with PPAR-γ ligand binding domain (PDB 3B0R) suggested a covalent binding mode in which the phenyl of **39** is covalently bound to the Cys285 through a S_NAr displacement (Fig. 14)[102]. In addition to covalent interaction, the amide group of the compound forms three hydrogen bonds with Cys285, Ser289 and Tyr327. The nitro group also interacts with two histones through hydrogen bonds. These extensive hydrogen bonds help the compound recognize and locate the polar pocket to specifically form covalent interaction with Cys285.

Liu et al.[103] reported that a selective mitochondrial phosphatase Ptpmt1 inhibitor alexidine dihydrochloride (**42**, Fig. 15) increased functional HSC numbers and promoted HSC long-term reconstitution capacity *via* activation of AMPK signaling. Furthermore, metformin was also reported to maintain HSC through similar mechanism with **42**.

4.2.7. *OCT4 agonist*

Transcriptional factor octamer binding protein 4 (OCT4) belongs to the POU (PIT/OCT/UNC) homeodomain protein family. OCT4 is responsible for maintaining self-renewal and pluripotency of human embryonic stem cells (ESCs) *via* a modulation circuitry involving SOX2 and NANOG[104]. Also of note, in some conditions, OCT4 allows the direct conversion from fibroblast to hematopoietic progenitors[105]. In 2016, researchers used a known OCT4 agonist OAC1 (**43**, Fig. 16) to support robust *ex vivo* expansion of human CB $CD34^+$ cells through downstream HOXB4 suppression[106]. A 2.8-fold increase in number of the most primitive HSCs was observed. Treatment of **43** also resulted in a 6.3-fold increase in SRC numbers which could long-term repopulate in NSG mice and secondary recipients without any leukemia potential.

4.2.8. *c-MPL agonist*

Thrombopoietin (TPO) receptor c-MPL plays an important role in HSC self-renewal and expansion. Nashino et al.[107] reported a small molecule c-MPL agonist NR101 (**44**, Fig. 16) which induced more than two folds expansion of $CD34^+$ subpopulation and about two-fold increase in SRC numbers in culture. NR-101 resulted in sustaining activation STAT5 and accumulation of HIF-1α protein.

Eltrombopag (**45**, Fig. 16) is a second generation marketed small molecule for treatment of chronic immune thrombocytopenia (ITP) and severe aplastic anemia (SAA) *via* binding and activating c-MPL[108]. A recent study by Kao and coworkers[108] gave insight into novel mechanism that eltrombopag enhanced hematopoiesis in a TPO-independent fashion but rather mediated by its iron chelation effect. In their study, eltrombopag reduced chelatable iron concentration in HSC, associated with decreased cellular reactive oxygen species (ROS) levels, subsequently to elicit relevant genomic profile alteration such as energy metabolism. They found HIF-1α was also increased in the context of low chelatable iron, which is an important regulator for HSC maintenance and self-renewal. Interestingly, the cation chelation effect for eltrombopag is similar to that of TEPA, which has been on clinical trials as an effective copper chelator for enhancing HSC expansion as previously described.

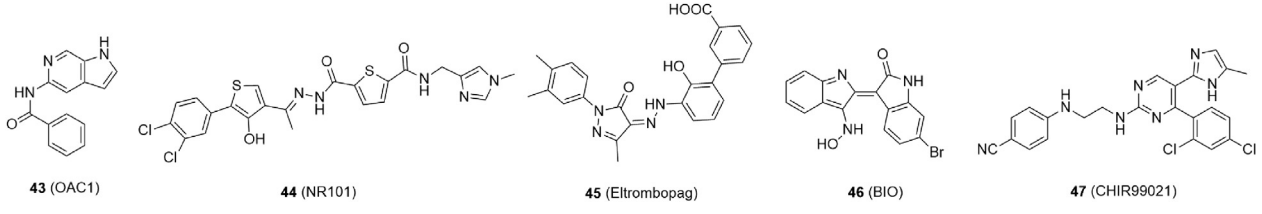

Figure 16 Structures of **43–47**.

4.2.9. GSK-3 inhibitor

GSK-3 is responsible for phosphorylation of a variety of protein, and therefore plays a pivotal role for signaling transduction in many pathways critical for HSC fate determination, such as Wnt, Notch and Hedgehog signaling[109]. Direct inhibition on GSK-3 elicits complicated downstream molecule events and positively regulates HSC self-renewal or maintenance[109]. Great efforts have been made to explore potent GSK-3 inhibitors and cooperation with other agents for valid HSC expansion in culture. Interestingly, prior to identification as a GSK-3 inhibitor, lithium has been used to ameliorate hematopoiesis and enables increase in $CD34^+$ cells and platelets in patients' peripheral blood[110]. Strategies of targeting GSK-3 using small molecules have been effective and been explored a lot.

Ko et al.[111] reported that treatment with BIO (**46**, Fig. 16) prolonged cell cycle and increased numbers of human CB HSC. Up-regulated CDK inhibitor p57 and down-regulated cyclinD1 may account for delayed cell cycle upon treatment with **46**. Although pharmacological inhibition on GSK-3 favored embryonic stem cells maintenance *via* activation of Wnt signaling, **46** did not modulate Wnt pathway but rather regulated Notch and Angpt1/Tie2 to promote HSC expansion and repopulating capacity in NSG mice.

Trowbridge et al.[109] reported that *in vivo* administration of a GSK-3 inhibitor CHIR99021 (**47**, Fig. 16) enhanced hematopoietic recovery in NSG mice, as a result, indicating a role for CHIR99021 in HSC maintenance and expansion. They employed combination of PI3K/AKT signaling agonist insulin and **47** to pharmacologically repress differentiation and promote self-renewal, yielding 100-fold transplantable HSCs. Later in 2012, Huang et al.[112] followed reporting another combination of mTOR inhibitor rapamycin (**54**, Fig. 18) and **47**, which supported maintenance of functional LT-HSCs and improved their repopulating ability in NSG mice. Arrest on cell cycle status without affecting apoptosis or death accounted for this expansion. Similarly, Li and colleagues[113] reported using a combination of **47** and a p38 inhibitor SB203580 could promote human UCB HSCs expansion *in vitro*.

4.2.10. Epigenetic modulators

Manipulation on histone protein or genomic DNA by small molecules is capable of regulating chromatin status, which is critical for DNA accessibility for replicate and transcription[114,115]. Modification by either de/methylation or de/acetylation greatly affects the interaction between adjacent nucleosome units by alteration of charge property of residues. Genomic alteration by epigenetic modification plays an important role for HSC cell fate decision[116,117]. All these modifications are "writed" or "erased" by corresponding enzymes. Regulating these enzymes with small molecules for HSC expansion has been really remarkable these years.

4.2.10.1. Histone acetyltransferases (HATs) inhibitor.

Nishino et al.[118] screened from 92 natural products and first found garcinol and isogarcinol (**55** and **56**, respectively, Fig. 18) were capable of expanding HSC. Both compounds are non-selective histone acetyltransferases (HATs) inhibitors. Garcinol enabled over 4.5-fold expansion of human CB HSC, and a 7.4-fold expansion was observed with isogarcinol treatment. In addition, garcinol increased SRC numbers in NSG mice by 2.5-fold. Gene expression profile showed enhanced expression of hepatic leukemia factor (*HLF*) and reduced *p53* level, both of which are important for self-renewal[119,120], despite no changes in well-established self-renewal genes, such as *HOXB4*, *BMI1*, *GATA2*, *Notch1*, *p21*, *p27*, *MYC*, *EGR*, and *EVI-1*.

4.2.10.2. Histone deacetylase (HDAC) inhibitor.

As an HDAC inhibitor, valproic acid (VPA, **48**, Fig. 17) has been widely used for treatment of epilepsy and other neurologic diseases. In 2005, two research groups both reported that VPA induced HSC proliferation and self-renewal and delayed cell cycle without inducing apoptosis, more importantly, maintained HSC both in short-term and long-term[121,122]. Hoffman's team demonstrated that HSC expansion induced by VPA treatment was accompanied by phenotypic transformation and cellular reprogramming of HSC[123–126]. VPA treatment promoted HSC multi-lineage reconstitution in serial transplantation in NSG mice[126–128]. VPA promoted HOXB4 and AC133 expression and histone acetylation level on their regulatory sites[121,122]. In addition, combination of VPA and GSK-3 inhibitor lithium preserved HSC stemness, repressed differentiation and enhanced hematopoietic potential[129].

Recently, Huang et al.[130] used a selective HDAC5 inhibitor LMK235 (**49**, Fig. 17) to promote human CB HSC homing. HDAC5 inhibition promoted p65 acetylation to result in CXCR4 upregulation in HSC surface, favoring HSC homing as well as engraftment to bone marrow niche *via* SDF-1/CXCR4 axis.

4.2.10.3. DNA methyltransferase inhibitor.

Early studies have indicated combination of 5azaD (**50**, a DNA methyltransferase inhibitor, Fig. 17) and trichostatin A (TSA, **57**, a histone deacetylase inhibitor, Fig. 18) altered HSC fate determination *in vitro*[131]. In 2006, Araki et al.[132] confirmed the role of **50** and **57** for enhancing CB HSC repopulating capacity in NSG mice. Later in 2007, they suggested that combination of treatment of **50** and **57** led to slow rate of cell division and cell cycle of human HSC, and these compounds preserved HSC hematopoietic potential in immunodeficient mice even if undergoing serial cell division[133,134]. Self-renewal implicated genes such as *HOXB4*, *BMI1*, *Notch1* and *GATA2*, and cell cycle inhibitors *p21* and *p27*, were all found to up-regulated in cells upon **50/57** treatment.

4.2.11. Cysteine protease inhibitor

Over the course of HSC expansion in culture, apoptosis is an important cause for HSC pool depletion which leads to subsequent expansion failure. Sangeetha and coworkers[135] employed two cysteine protease inhibitors zVADfmk and zLLYfmk (**51** and **52**,

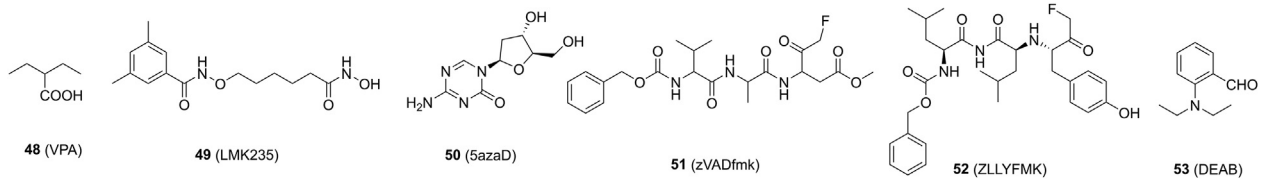

Figure 17 Structures of **48–53**.

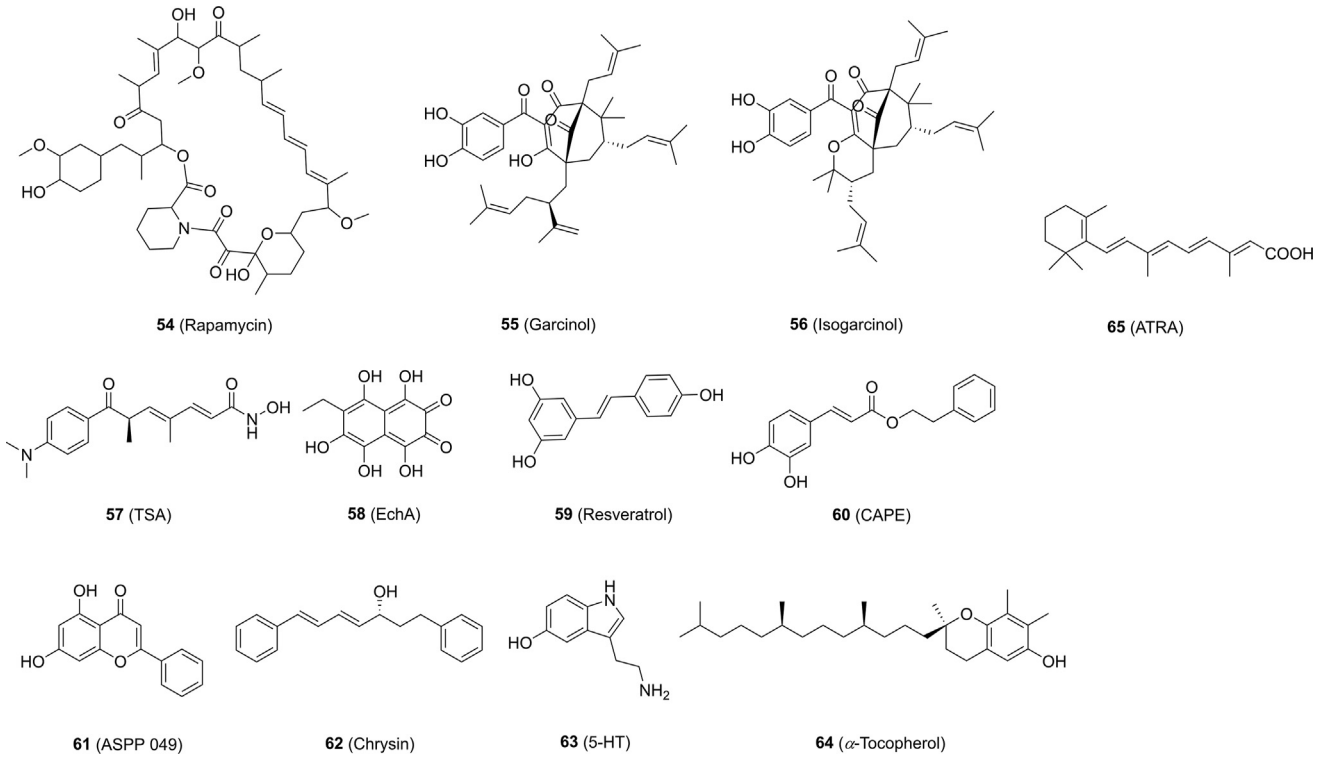

Figure 18 Structures of natural products and endogenous substances for HSC expansion.

Fig. 17) to suppress HSC apoptosis and observed increased CB $CD34^+$ cell numbers and enhanced their engraftment activity. This anti-apoptosis effect was possibly mediated by bcl-2, caspase-3 and Notch signaling.

4.2.12. Aldehyde dehydrogenase (ALDH) inhibitor

Aldehyde dehydrogenase (ALDH) is responsible for converting retinol (vitamin A) to retinoic acid and is highly enriched in HSCs. Chute et al.[136] demonstrated that ALDH plays an important role for HSC differentiation and an ALDH inhibitor DEAB (**53**, Fig. 17) induced robust *ex vivo* expansion of CB HSCs and 3.4-fold increase in SRC numbers relative to uncultured counterparts. **53** delayed HSC differentiation and promoted self-renewal *via* inhibiting retinoic acid production. Consequently, modulation on retinoid signaling may be a promising strategy for HSC expansion.

4.3. Natural products and endogenous substances

4.3.1. Natural products

Naturally occurring products are an important source for exploring novel agents for *ex vivo* HSC expansion. While natural active products are to be blamed for over-extensive biological activities and elusive molecular mechanisms, novel molecular scaffolds and exclusive effect from natural products have been at times to give researchers insight into drug discovery. In addition to HATs inhibitor garcinol and HDAC inhibitor trichostatin A described above, echinochrome A (Ech A, **58**, Fig. 18) has recently been reported for *ex vivo* HSC expansion[137]. In this study, **58** induced 1.33-fold expansion of $CD34^+$ cells and 1.7-fold CFUs increase in culture over control *via* Src/Lyn activation, ROS inhibition and activation of p110δ in PI3K/AKT. Resveratrol (**59**, Fig. 18) is a natural polyphenol and has broad-spectrum biological functions and health benefits. Apart from promoting hematopoiesis *in vivo*[138,139], resveratrol also improved *ex vivo* expansion of CB HSC and enhanced engraftment of expanded HSCs in primary and secondary NSG mice recipients[140].

Similar to Ech A, cafeic acid phenethly ester (CAPE, **60**, Fig. 18) also acts as an antioxidant, derived from propolis. **60** promoted *ex vivo* CB-HSCs expansion, homing and engraftment *in vivo*, mediated by up-regulation of HIF-1α and vascular endothelial growth factor-A (VEGF-A)[141,142].

Recently, researchers from Thailand reported a diarylheptanoid compound ASPP 049 (**61**) isolated from *Curcuma comosa* with *ex vivo* expansion activity and repopulating capacity *in vivo*[143]. RNA-seq analysis of expanded HSCs suggested Hippo pathway was most affected.

Chrysin (5,7-dihydroxyflavone, **62**, Fig. 18) is a naturally occurring flavone, mainly found in passion flowers, honey, and propolis. It acts as an antioxidant to exert anti-tumor effects in several cancers, with several signaling been affected, such as MAPK and PI3K/AKT[144]. Screened from 85 antioxidant small molecules, chrysin was identified to expand HSC effectively and promote rapid engraftment in NOG mice, which might be attributed to its antioxidant-effect[145]. All discussed natural products are summarized in Table 3.

4.3.2. Endogenous substances

5-hydroxytryptamine (5-HT, **63**, Fig. 18), also termed serotonin, is an endogenous neurotransmitter widely involved in nerve conduction. Yang et al.[146] reported 5-HT enabled CB HSCs expansion in culture and increase in CFUs. More importantly, long-term repopulating capacity of these expanded HSCs was augmented. Mechanistic studies suggested 5-HT inhibits apoptosis and protects from mitochondria damage.

α-Tocopherol (**64**, Fig. 18) is one of vitamin E compound family members. Nogueira-Pedro et al.[147] reported its special role

in enhancing murine HSC activity *in vivo*, however, accompanied by ERK1/2 mediated differentiation. α-Tocopherol treatment induced 2-fold expansion in murine LSK cells.

As a derivative of vitamin A, all-*trans* retinoic acid (ATRA, **65**, Fig. 18) is an endogenous agonist of RA receptor. Purton et al.[148,149] reported ATRA enhanced mouse HSC repopulating and self-renewal ability involving Notch1 and HOXB4 activation, whereas contradicting effect occurred when ATRA treatment could reverse ALDH inhibition-induced enhancement of HSC self-renewal capacity[136].

5. Potential targets for HSC *ex vivo* expansion

5.1. MLLT3

Ubiquitous histone modification process plays an essential role for DNA transcription, repair and replication by means of manipulation of DNA activity state[150]. MLLT3, also known as AF9, recognizes and binds to histone H3K9 acetylation site in transcription start sites *via* its YEATS domain, and recruits DOT1L to methylate H3K79 lysine for regulating target gene expression (Fig. 19A)[151,152]. A recent study indicated that MLLT3 is enriched in human HSC and is responsible for human HSC maintenance and repopulating capacity through promoting transcription of self-renewal related genes[14]. MLLT3-overexpression (MLLT3-OE) significantly enhanced CB HSC *ex vivo* expansion in either stromal stem cell co-culture condition or culture supplemented with SR1 and UM171. Limited dilution assay exhibited a 12.5-fold increase in transplantable HSC of MLLT3-OE HSCs relative to that of uncultured HSCs, and 5.2-fold higher than control vector. Sustaining MLLT3 overexpression in CB HSC in culture promoted stable multilineage reconstitution in primary and secondary recipients. Moreover, MLLT3-OE did not result in any lineage differentiation bias, aberrant proliferation or apoptosis, suggesting its well safety profile.

Targeting this essential self-renewal regulator with specific small molecules in culture conditions may give hope for stable *ex vivo* HSC expansion.

5.2. Musashi-2

RNA binding protein-mediated post-transcription modification occurred in many aspects during the course of HSC self-renewal and differentiation, affecting a range of relevant mRNA translation[153]. RNA binding protein Musashi-2 has been implicated as an oncogenic factor in many types of cancer, for example, involvement of Musashi-2 in AML patients was usually associated with poor prognosis[154]. In normal HSCs, Musashi-2 is an important regulator for HSC maintenance and self-renewal[155,156]. Musashi-2 deficient mice were accompanied by hematopoiesis defect, and deletion of *Musashi-2* in HSC led to loss of repopulating capacity[155,156].

In 2016, Rentas and colleagues[157] identified the role for Musashi-2 in HSC self-renewal, whose overexpression resulted in 17- and 23-fold increase in ST-HSC and LT-HSC, respectively. Musashi-2 overexpression promoted human CB HSPCs self-renewal and their engraftment in NSG mice, in contrast to Musashi-2 knockdown. Mechanistic studies suggested that Musashi-2 regulated post-transcriptional processes *via* inhibition of AHR signaling for promoting HSC self-renewal and *ex vivo* expansion, similar to SR1.

In addition to targeting on upstream signaling for Musashi-2 overexpression, another potential strategy is to activate RNA binding pocket or allosteric site with small molecule. There have been reported small molecules for targeting its RNA binding pocket[158,159], which could provide some insight into developing potential Musashi-2 agonist.

5.3. YTHDF2

In all eukaryotes, N^6-methyladenosine (m^6A) is the most abundant epigenetic modification occurring in numerous mRNAs[160]. As a post-transcriptional modification way, m^6A is associated with HSC specification in embryogenesis[161], differentiation[162], as well as leukemogenesis[163]. m^6A regulates mRNA translation and decay by virtue of "writer" METTL3/METTL14, "eraser" FTO/ALKBH5 and "reader" YTHDF1−3[164]. Among m^6A readers, YTHDF2 could recognize many m^6A tagged mRNAs by its conserved aromatic cage[165,166] and degrade them through subsequent recruitment of deadenylase complex[167]. Recent studies disclosed that depletion of YTHDF2 stabilized those m^6A-marked mRNAs encoding for transcription factors which are critical for self-renewal in mouse HSPCs and human UCB HSCs, and as a result promoted *ex vivo* expansion of HSCs without any noticeable lineage bias or leukemic potential[13,168]. In addition, researchers from the University of Edinburgh indicated that YTHDF2 is dispensable for normal HSC activity but required for leukemic stem cells in AML[169]. These investigations demonstrate that YTHDF2 would be a promising target for obtaining sufficient transplantable HSCs *in vitro*, whereas the first YTHDF2 small molecule inhibitor has not yet been identified.

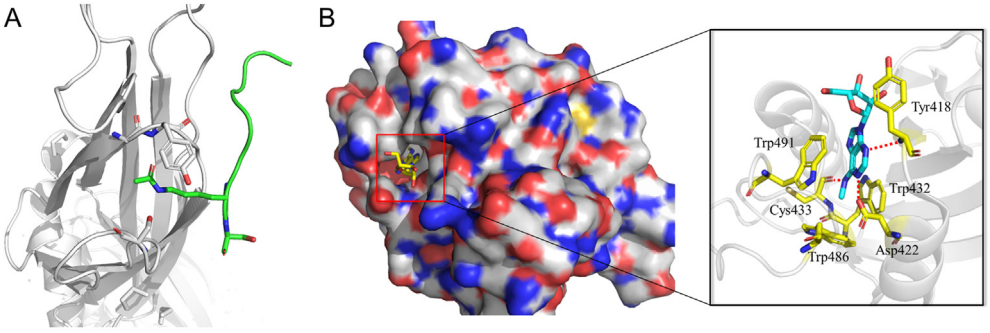

Figure 19 (A) Recognition mode of AF9 YEATS domain to H3K9 peptide (green sticks) (PDB 4TMP). (B) Protein surface of YTHDF2 in complex with m^6A mononucleotide and close up view of interaction between m^6A mononucleotide and residues of YTHDF2 protein (PDB 4RDN). H-bonds are depicted in red dashed line, and key amino acid residues are labeled in black text.

Crystal structure of YTHDF2 in complex m^6A mononucleotide may give some insight into design of its inhibitors (Fig. 19B). YTHDF2 recognizes m^6A through a conserved aromatic cage which is formed by three aromatic amino acid residues Trp432, Trp486 and Trp491. Three hydrogen bonds are formed between adenosine ring moiety and Tyr418, Asp422 and Cys433 residues. $\pi-\pi$ stacking also generates between the adenosine ring and adjacent aromatic residues. To inhibit YTHDF2, heterocycle bearing sufficient H-bond donors is required for molecule−protein interaction. As the aromatic pocket is small and hydrophobic, substitutions on heterocycle are expected to small and nonpolar.

5.4. *RET*

The neurotrophic factor receptor RET was reported to express in HSC surface and mediate HSC survival, normal differentiation potential and repopulating capacity[170]. Activation of RET with glial derived neurotrophic factor (GDNF) family ligands (GFLs), which are secreted in HSC niche, promotes HSC expansion *in vitro* and transplantation efficiency *in vivo*[170,171]. Moreover, upon RET activation, a lot of signaling pathways, such as p38-MAPK, CREB, AKT, ERK1/2, NFκB and p53, were driven to collectively improve HSC expansion and repopulating activity. These findings may inspire the exploration of RET small-molecule agonists for human HSC *ex vivo* expansion and cord blood transplantation.

6. Summary and perspectives

As an alternative source of HSC, cord blood has several advantages such as easy availability, tolerance of partial HLA-mismatch and low incidence of chronic GVHD[5]. However, low stem cell dose limits the use of CB unit for transplantation to pediatric patients[6]. *Ex vivo* HSC expansion is an effective method for obtaining sufficient HSCs. Currently, although extensive efforts have been devoted to every approach, stable HSC expansion *in vitro* have been yet unattainable. The biggest hurdle laid ahead *ex vivo* HSC expansion is to balance self-renewal and differentiation in culture. Little is known about the definite molecule mechanisms of HSC self-renewal and why they fail *in vitro*. The answer for these queries may be found where HSC lives. Briefly, HSC lives in a bone marrow microenvironment supported by various cells. Diverse niche cell types and growth factors are involved in regulating HSC self-renewal and differentiation[17,21,22]. Stable and sustaining *ex vivo* HSC expansion may be available when all niche components and their molecular mechanism are fully elucidated.

In fact, the balance between self-renewal and differentiation *in vivo* is strictly controlled by delicate extracellular molecule cues and complex intracellular signaling pathways such as two conserved Wnt and Notch signaling[26,27]. Rapid self-renewal or inadequate differentiation pose risks of leukemic transformation, while inadequate self-renewal or rapid differentiation lead to HSC pool exhaustion[53]. Early to about twenty years ago, genetic manipulation of HSC such as HOXB4 gene led to hundred folds increase in self-renewal capacity[172]. Nevertheless, it is risky for patients to receive these HSCs which may lead to leukemia. As a result, transient and removable activation by extrinsic agents is desirable for *ex vivo* HSC expansion[53]. Early attempts begin with identification of a variety of cytokines or growth factors and cocktails of them. Combination of SCF, TPO, FLT-3L and IL-6 is a commonly used cytokine cocktail for HSC expansion culture. In addition to these classic cytokines, more developmental growth factors were identified for HSC expansion such as FGFs, IGFs, IGFBPs, Angptls, Shh, BMP-4, Wnt proteins and Notch ligands[53]. All of them support considerable HSC expansion while maintain long-term repopulating capacity. In 2010, Delaney et al.[50] reported clinical outcomes of a phase I trial of Notch ligand-expanded CB unit. Rapid neutrophil recovery (main indicator for graft engraftment) was observed with a median time of 16 days. However, attempts to explore cytokines or growth factors for HSC expansion are overall unsuccessful because inevitable differentiation occurs commonly after transient expansion, which suggests additional agents are necessary[53].

Compared to cytokines, small molecules have significant advantages such as having definite targets and effects, easily manipulated *in vitro* and low cost, which make them valuable candidate agents for clinical therapy in the future. As a result, people turn their sights to promising small molecules and great achievements have been made. Since the first small molecule TEPA was evaluated preclinically and in a phase I clinical trial during the first decade of this century, the last decade have seen rapid discovery and development of more potent and efficacious small molecules, such as SR1, PGE2, nicotinamide and UM171. All these small molecules significantly expand HSC numbers in CB unit and provide rapid hematopoietic recovery in patients compared to those unmanipulated units, with median time to neutrophil recovery generally shorter than 15 days and without any serious adverse events. However, rapid engraftment appears to not benefit disease-free survival, possibly expanded HSCs losing long-term reconstitution capacity *in vivo*.

Early attempts and exploration for small molecules such as GSK-3 inhibitors, epigenetic modulators laid out the foundation for finding more potent small molecules and novel targets[109,111,132]. Some endogenous substances such as 5-HT, α-tocopherol, and retinoic acid were all reported having HSC expansion activity[146,147,149]. In addition, many natural products such as garcinol, echinochrome A, and resveratrol were used to expand HSC and pleasant results were obtained[118,137,139].

However, lineage commitment at the cost of HSC depletion commonly occurs when targeting on single signaling or target alone. Thus, regulation of single signaling or target may be not sufficient for multi-dimensional regulation of cellular processes to obtain stable *ex vivo* HSC expansion, because complex molecule signaling orchestrates HSC fate decision. For example, HSCs expanded by VPA underwent phenotypic transformation and cellular reprogramming involving mitochondrial remodeling and p53 activation[123]. As an integration of complex cellular events is required for *ex vivo* HSC expansion, it is possibly that the optimal condition for HSC expansion is combination of several small molecules. A cocktail of SR1, CAY10433 and VPA was reported to support ~20-fold expansion of $CD34^+$ cell and retain multilineage potential of these HSC *in vivo*[173]. Similar result was also observed in a cocktail of CHIR-99021, forskolin and OAC1[174]. A combination of GSK-3β inhibitor CHIR99021 and a p38 inhibitor SB203580 was also used to robustly expand HSC *in vitro*[113].

As is so successful that small molecules have made great landmark for HSC expansion, however, currently almost all clinical trials restricted to evaluate two CB units (one expanded, the other unmanipulated). Therefore, in some context, effect of small molecules may be elusive, possibly due to the unmanipulated CB unit. In fact, in many cases, long-term engraftment derived from

the expanded unit completely lost after several months. There have been two reasonable explanations[50−52]: (1) HSCs in the expanded unit are only responsible for short-term repopulation, and long-term HSCs gradually deplete *in vivo*. (2) Immune cells present in the unmanipulated unit produce an immune rejection against the expanded unit, and the former becomes the winning unit. Moreover, in some studies[63,78], small molecules appeared not to produce significant positive outcomes in terms of overall survival and disease-free overall survival, despite rapid neutrophil recovery or low incidence of GVHD. Clinical studies based on single expanded CB unit have proceeded slow and these outcomes are not desirable[8,9,64]. We suppose these issues will be handled well as we get a comprehensive knowledge about HSC in the future. Perhaps, the true answer for *ex vivo* HSC expansion is not small molecules alone, but a combination of multiple approaches which need multidisciplinary struggle.

Applications of new technologies and methodologies have allowed for high efficiency in discovery of novel small molecules and targets. Additionally, the discovery and development of small molecules for HSC expansion really requires an integration of interdisciplinary cooperation, such as chemistry, biology and computer science. In recent years, many novel and potent small molecules have been identified by means of new technologies, such as high throughput screening (HTS), computer-aided drug design (CADD) and artificial intelligence (AI). Furthermore, prompt developments in synthetic chemistry have benefited and allowed for large-scale preparation of desired compound for further animal experiment and clinical trials, so did combinatorial chemistry enable rapid and convenient establishment of a huge library of small molecules for HTS. Traditional medicinal chemistry strategies involving SAR studies and rational drug design provide optimization strategy for lead compound to obtain better potency and druggable properties. In terms of HSC expansion, as exemplified by eltrombopag, drug repurposing strategy enables the availability of clinical approved drug for new diseases or therapies with significantly reduced time-waste and cost. Covalent inhibitors such as JNK-IN-8 and GW9662 were reported to support HSC expansion *via* covalent interaction with targets. p18 inhibitors were identified by *in silico* screening based on known p18/CDK6 complex crystal structure and optimized by SAR studies[95,98]. Solubility of 15-PGDH inhibitor SW033291 was improved by 10,000-fold through SAR studies[79,81]. In addition, several novel and safe targets for *ex vivo* HSC expansion have been reported recently, such as YTHDF2 and MLLT3[13,14].

The field of HSC expansion has enjoyed a banner decade, with a number of potent small molecules and several novel biological targets for *ex vivo* expansion of HSC intensively developed. It is conceivable that, as a branch of regenerative medicine, sufficient HSCs expanded by small molecules or other approaches will be clinical availability for treatments of human diseases in the near future.

Acknowledgments

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Author contributions

Conception and design: Jiaxing Li, Xiao Wang, Peng Yang. Acquisition, analysis and integration of data: Jiaxing Li, Xiao Wang, Jiayu Ding, Wenjian Min, Yasheng Zhu, Wenbing Kuang, Kai Yuan, Chengliang Sun, Peng Yang. Writing-review and editing: Jiaxing Li, Xiao Wang, Peng Yang. Administrative, technical, or material support: Xiao Wang, Peng Yang.

Conflicts of interest

The authors declare no competing financial interests.

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