Introduction

Acute myeloid leukemia (AML) is a malignant disorder, consisting of a heterogeneous group of clonal hematopoietic stem cell neoplasms. AML is characterized by aberrant accumulation of immature myelocytes in the bone marrow, which interferes with the production of normal blood cells. The last four decades have seen great advances in treatment-optimization and supportive care. However, long-term effective cure has been by far undefined (1). After being treated with chemotherapy, most patients can achieve complete remission (CR), but only about 20-25% patients remain long-term disease-free survival (DFS) (obtained from the NCI's SEER database) (2). It becomes even worse for elderly patients (>60 years); the figure drops down to 10-15% only. It is therefore not surprising that the death rate can reach as high as 50% for young adult patients and 90% for elderly AML patients (3). Low DFS is largely explained by refractoriness to chemotherapeutic strategies and relapse after CR, both of which become the main bottlenecks in current AML therapy.

The high frequency of drug resistance and relapse after conventional chemotherapy is a result of failure of targeting leukemic stem cells (LSCs). Accumulating evidences have supported that LSCs are key drivers of leukemia initiation, progression and relapse. Similar to normal hematopoietic stem cells (HSCs), LSCs exhibit stem cell-like characteristics such as the capacity for self-renewal, multipotent differentiation potential and relative quiescence (4). Higher expression of multi-drug resistance (MDR) proteins renders LSCs resistant to conventional chemotherapeutic agents. The persistence of surviving LSCs is responsible for the high frequency of relapse (5-8). Undoubtedly, successful therapeutic strategies should also eliminate these LSCs. It is therefore crucial that therapies be developed targeting the quiescent and drug-resistant LSCs. To name but a few, they include: (I) the abnormal expression of CD markers (e.g., CD44, CD47, CD96, and CD123) (9-12); (II) constitutive activation of nuclear factor kappa B (NF-κB) (13); (III) active Wnt/
β-catenin signaling (14); (IV) elevated levels of interferon regulatory factor-1 (IRF-1) and death-associated protein kinase (DAPK) (15); and (V) the sensitiveness to reactive oxygen species (ROS) homeostasis (16-18). Thankfully, these characteristics may provide important hints for designing LSC-targeted therapy.

In this review, we mainly focus on ROS and their roles in AML therapies, especially in targeting AML stem cells. ROS are a heterogeneous group of small molecules, including super-oxide, hydrogen peroxide, singlet oxygen, ozone, hypo-halous acids and organic peroxides (19-21). Due to highly chemically reactive nature, it is much easier for ROS to damage DNA, proteins and lipids than molecular oxygen does. In hematopoietic cells, ROS are largely generated in the mitochondria, through nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and other ROS-related metabolic pathways (e.g., polyamine metabolism, cytochrome P450 and xanthine oxidase) (22). In hematological malignancies, oxidative stress overload (due to increased ROS) has been identified in several other myeloid neoplasms in addition to AML, thus representing a universal feature in myeloid diseases (23,24). Several studies have reported the association between increased levels of oxidative stress and AML relapse, indicating that ROS over-production may be a critical factor in AML initiation and progression. More importantly, ROS may be an optimal therapeutic target for AML-stem cells therapy. This is evident in our recent report and many others; ROS-generating agents can selectively eradicate AML-stem cells via modulating ROS production (25-34).

Pathologic features of AML stem cells

LSCs account for a rare sub-population of leukemia cells, but are the real instigator of currently poor prognosis in AML therapies. There is a hot debate on their origin, but a consensus being reached is that LSCs do not come from single source; they can originate either from long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs), or multi-potent progenitors (MPPs), or even more differentiated progenitors [i.e., common myeloid progenitors (CMP) and granulocyte/macrophage progenitors (GMP)] (35). Similar to normal HSCs, LSCs exhibit stem cell-like characteristics such as the capacity for unlimited self-renewal, impaired hematopoietic differentiation and the ability of generating heterogeneous leukemia cells that comprise leukemia. However, the signaling pathways controlling “stemness” in LSCs are not exactly the same as those in HSCs. These pathways are disturbed in LSCs. For example, self-renewal associated pathways [B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1), telomerase and signal transducer and activator of transcription 5 (STAT5)] (36-39) and developmental pathways [Notch, Wnt/β-catenin and sonic hedgehog (Shh)] (14,40,41) are aberrantly activated. Other miscellaneous pathways related to apoptosis, differentiation and drug resistance [NF-kB, fms-like tyrosine kinase-3 (FLT3), phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and P-glycoprotein (P-gp)] are constitutively activated (8,13,42-45). Recent studies have also shown unique epigenetic pathways requisite for maintaining AML-stem cells self-renewal, including highly expressed histone H3 lysine 36 dimethyl-specific demethylase KDM2b/JHDM1b (46). In addition to these pathways, LSCs also have specific immunophenotypic markers, including CD117, CD82, CD47, CD96, CD32, CD33, CD25, Cyclin-A1, H-Y antigens, C-type lectin-like molecule-1 (CLL-1), T cell immunoglobulin mucin-3 (TIM3) and CD44 (10,11,47-56). The greatest difference is their unique location in bone marrow. Most LSCs are concentrated within the osteoblast-rich area. This area is of low oxygen pressure and is highly immune to cytotoxicity induced by chemotherapy. Under low-oxygen condition, AML-stem cells are arrested at stable quiescent cycling stage, and over-express a higher P-gp, MDR-related protein 1 (MRP), breast cancer resistance protein (BRCP) and lung-resistance protein (LRP) (8). The quiescence and higher MDR expression confer drug-efflux capabilities on LSCs to escape from chemotherapies.

ROS homeostasis

The generation source of ROS

Intracellular ROS can be produced exogenously and endogenously. The exogenous sources of ROS include smoke, air pollutants, ultraviolet radiation, γ-irradiation, and many chemicals able of generating ROS. The endogenous sources of ROS are much diverse. It can come from mitochondria, a family of NADPH oxidases (NOXs), and ROS-related metabolisms (57). The mitochondrial respiratory system is believed to be the major intracellular source of ROS. Upon pro-oxidant stimuli, ROS (e.g., O2·−, HO·) are generated by accompanying unstable mitochondrial membrane potential (ΔΨm), the electron delivery through the mitochondrial respiratory complexes and proton gradient establishment across the inner mitochondrial membrane.
These metabolisms are usually catalyzed by cytochrome P450 enzymes, polyamine and amino acid oxidases, xanthine oxidase, lipoygenases, cyclooxygenases, flavoenzyme ERO1 in the endoplasmic reticulum (ER), flavin-dependent demethylase, and nitric oxide synthases, to name but a few (58-60). Other sources include free iron or copper ions, haem groups, and metal storage proteins responsible of converting \( \text{O}_2^- \) and/or \( \text{H}_2\text{O}_2 \) to \( \text{OH}^- \).

**The scavenger system of ROS**

Cells have evolved an elaborate defense system to scavenge excess ROS so as to avoid oxidative stress damage. This scavenger system is important to maintain ROS homeostasis, especially for hematopoiesis (61). To reach oxidation-reduction (redox) equilibrium, a variety of cellular antioxidant enzymes and non-enzymatic molecules can be utilized. The enzymatic antioxidant enzymes include uperoxide dismutases, catalases, glutathione peroxidases, glutathione reductase, thioredoxins, thioredoxin reductases, methionine sulfoxide reductases, peroxiredoxins and peroxynitrite reductases. The non-enzymatic molecules include ascorbate, prruvate, \( \alpha \)-ketoglutarate (\( \alpha \)-KG) and oxaloacetate; all these can directly react with ROS for which is kept at a homeostatic level.

**The influential roles of ROS in HSCs**

It has long been recognized that ROS play a critical role in balancing self-renewal and myeloid differentiation of HSCs. Studies using animal models has demonstrated that coordinated regulation of ROS levels is of great importance for HCS quiescence (62,63). Mice lacking the ataxia telangiectasia mutated (ATM) can develop bone marrow failure that is correlated with increased levels of ROS (64). The impairment of HSCs in ATM-/- mice is due to the aberrant activation of p38 MAPK signaling pathway caused by increased ROS (65). The forkhead O (FoxO) family of transcription factors is important to control ROS levels of quiescence HSCs compartment (66). Mice with conditional loss of FoxO1, FoxO3, and FoxO4 suffer from decreased number of HSCs, impaired capabilities of hematopoietic reconstitution in competitive and noncompetitive recipient mice. However, increased levels of ROS induce differentiation of HSCs into myeloid progenitors but in a manner independent of FoxO activity (67,68). The findings above in mice are also validated in fruit flies: the accumulation of ROS is an important factor triggering myeloid progenitor differentiation from HSCs (67).

Mechanistic studies reveal that increased levels of ROS induce HSCs to exit from the niche for the differentiation via suppressing the expression of N-cadherin and reducing HSCs-osteoblast adhesion (63). However, abnormal robust ROS accumulation is closely associated with HSC senescence (69). ROS-induced HSC senescence is regulated by signaling pathways including FoxOs, ATM, mTOR, TSC1, Bmi1 and AKT. Other pathways such as p53-p21 and p16-Rb activation can also rapidly induce ROS increase and then trigger HSC senescence.

**The emerging roles of ROS in AML**

Cancer cells rely on glycolysis but not aerobic mitochondrial respiration for energy supply, and tend to live in the lower pressure of oxygen environment than their normal counterparts. Low level of intracellular ROS is even common when it comes to cancer stem cells (CSCs), regardless of their tissue origins (e.g., breast, gallbladder, liver, lymphoma and so forth) (70-75). It is likely a metabolic hallmark of CSCs. The knowledge about the association between ROS levels and cancer (and CSCs) is largely learnt from the studies on AML. First, some genetic abnormalities in AML are directly relevant to ROS metabolism. One of the most common genetic abnormalities is Ras [including 10% of neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) mutation, 2% of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation and rare v-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRAS) mutation], leading to constitutive activation of Ras signaling. CD34+ human hematopoietic progenitor cells with Ras mutations are capable of inducing ROS production (68). Genome-wide sequencing studies have identified 7% isocitrate dehydrogenase 1 (IDH1) and 8% isocitrate dehydrogenase 2 (IDH2) somatic mutations in AML patients. These phenotypic mutations impair the normal enzymatic activity of IDH1/2, leading to decreased production of NAD(P)H, \( \alpha \)-KG and glutathione, and increased production of oncometabolite 2-hydroxylglutarate (2-HG). These genetic abnormalities can disturb the balance between pro-oxidative and anti-oxidative states, which eventually increases the ROS production (76,77). Recent published data demonstrate that active FLT3 signaling (over 30% AML patients with FLT3 mutation) is also closely related to increased ROS production (24,78,79). Second, AML-stem cells tend to have a lower metabolic activity (thanks to their quiescent nature), thus a lower ROS production than the AML cells as a whole. Through delicate functional assays, Lagadinou
et al. demonstrate that the majority of AML-stem cells are enriched in the ROS-low population. Moreover, this study further shows that these ROS-low cells overexpress BCL-2, and are more sensitive than ROS-high counterparts to cell death induced by the BCL-2 inhibitor. The BCL-2 is an apoptosis antagonist, characterizing the apoptotic-resistance feature of AML-stem cells (31,80). Thanks to the ROS difference, different responses to BCL-2 inhibitors can be used for AML therapies.

Agents selectively induce AML-stem cells cell death via ROS

In this section, we provide a detailed review of ROS-generating agents that show potentials to target primary AML-stem cells (summarized in Table 1). ROS play a decisive role in eradicating AML-stem cells. Though the mechanisms of how ROS are produced by these agents remain unclear, increasing evidence suggest that impairment of the scavenge system might play the central role in ROS generation. This is exemplified by (I) 3-deazaneplanocin A (DZNep) up-regulates thioredoxin-binding protein 2 (TXNIP) to inhibit thioredoxin activity, which in turn causes ROS production (33); (II) treatment with ABT-263 causes decreased levels of reduced glutathione (GSH) in AML-stem cells (31); (III) antioxidants such as N-acetylcysteine (NAC), vitamin C or α-tocopherol can protect AML-stem cells from targeting (25,32,34); (IV) NF-κB, a key transcription factor for the redox balance, is inhibited by most AML-stem cells targeting agents (26,27,29,30,34). Besides ROS induction, other mechanisms mainly include NF-κB inactivation, p53 activation, FLT3 inhibition, Wnt inhibition, PKC inhibition and membrane disruption. Integration of ROS with other key stemness/survival signaling pathways brings powerful toxic efficacy on AML-stem cells (Figure 1). Until now, 11 agents have been verified in AML-stem cell targeting capabilities.

Bcl-2 inhibitors

Small molecule BCL-2 inhibitors, ABT-737 and ABT-263, have been shown to selectively kill ROS-low AML-stem cells (31). In vivo experiments reveal that BCL-2 inhibitors severely impair mitochondrial oxidative phosphorylation, rapidly deplete intracellular ATP production, and induce a robust generation of mitochondrial ROS. More importantly, there are no cytotoxic effects observed on ROS-high AML cells (including higher proliferate rate AML cells and AML blast cells). BCL-2 inhibitors have entered a phase I clinical trial for evaluating the clinical efficacy in AML/myelodysplastic syndrome (MDS) treatment. In this trial, the small-molecule pan-BCL-2 inhibitor, called ‘obatoclax mesylate’, was prescribed to 44 patients with refractory leukemia and myelodysplasia to assess its safety and optimal dose. One AML patient with mixed lineage leukemia t(9;11) rearrangement achieved a CR that lasted for 8 months. Three of 14 MDS patients showed hematologic improvement for less red blood cells or platelet transfusion dependence (81). This clinical trial comes to the conclusion that obatoclax mesylate is a well-tolerated pan-BCL-2 inhibitor with early efficiency, calling for next-phase trial for its eventual clinical use in targeting AML stem cells.

Parthenolide (PTL) and dimethylaminoparthenolide (DMAPT)

PTL is a naturally occurring small molecule that has been evaluated for in vitro and in vivo efficacy on AML progenitor and stem cell populations. However, low soluble feature makes its pharmacologic potential less attractive. Instead, DMAPT, a dimethylamino analog of PTL, demonstrates 1,000-fold greater solubility in water than PTL. Both of DMAPT and PTL show similar efficacy on AML-stem cells through the similar molecular mechanisms, including the elevated ROS, p53 activation and NF-κB inactivation. Canine xenograft experiments, an equivalent to phase I clinical trials, show that the pharmacologic properties of DMAPT are superior to PTL (26,27). These data call for further studies on clinical safety and translational efficacy of DMAPT/PTL in AML treatment.

Fenretinide

Fenretinide is a well-tolerated vitamin A derivative that lacks a carboxyl functional group likely necessary for retinoid receptor activity. Our most recent study has shown that it is capable of eradicating LSCs but not normal hematopoietic progenitor/stem cells, at physiologically achievable concentrations (5 μM). Fenretinide-induced AML-stem cells death is associated with the rapid generation of ROS, induction of genes responsible for stress responses and apoptosis, and repression of genes involved in NF-κB and Wnt signaling (34). Though there is no clinical trial ongoing for AML treatment, fenretinide has been verified its safety and low toxicity in phase II-III clinical trials for solid tumors such as small cell lung cancer,
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AML, acute myeloid leukemia; HNE, 4-hydroxy-2-nonenal; DZNep, 3-deazaneplanocin A; PTL, parthenolide; DMAPT, dimethylaminoparthenolide; TDZD-8, 4-benzyl, 2-methyl, 1,2,4-thiadiazolidine, 3,5 dione; ROS, reactive oxygen species; NF-κB, nuclear factor kappa B; GSH, glutathione; ER, endoplasmic reticulum; FLT3, fms-like tyrosine kinase-3.
breast cancer and prostate cancer (82–85). Moreover, via bioinformatics analysis we have observed that fenretinide down-regulated genes are significantly correlated with genes related to a poor prognosis/relapse of AML. We anticipate that fenretinide is a potent AML-stem cells targeting candidate in the treatment of AML.

Niclosamide

Niclosamide has antihelminthic activity specific to most tapeworms but not other worms (e.g., pinworms and roundworms), and has been approved for use by Food and Drug Administration (FDA) (30). Mechanistic studies have shown that it is able to inhibit the transcription and DNA binding of NF-kappaB, and block the tumor necrosis factor-induced signaling pathway. Interestingly, nicloamide also increases increases the levels of ROS in AML cells (especially in CD34+CD38− subpopulation), kills AML progenitor/stem cells and inhibits their AML repopulation capability in xenograft recipients. When used in combination with frontline chemotherapeutic regimens such as cytarabine, etoposide and daunorubicin, nicloamide has a synergistic efficacy. These combinations suggest multi-layer anti-leukemia effects, and deserve immediate studies to clarify chemo-sensitive potential and inhibition of AML repopulation in nude mice.

Celastrol and 4-hydroxy-2-nonenal (HNE)

Both agents can effectively eradicate AML at the bulk, progenitor, and stem cell level. They were first identified through an in silico screen method, called gene expression-based high-throughput screening (GE-HTS) and the Connectivity Map (29). The concept is based on gene expression signature reversal strategy for screening newly AML-stem cells targeting agents. In-depth experiments suggest that two agents share mechanisms including ROS induction and NF-kB inactivation. No clinical trials have been reported, and so there is no data for their safety and efficacy in AML treatment.

4-benzyl, 2-methyl, 1,2,4-thiadiazolidine, 3,5 dione (TDZD-8)

TDZD-8 is a non-ATP competitive inhibitor of GSK-3β. It is widely studied as a cytoprotective agent to treat septic and nonseptic shock, lung injury, arthritis, spinal cord injury, colitis and Alzheimer disease. Guzman et al. explores another functional facet of TDZD-8, namely selectively killing AML-stem/progenitor cells. Cellular and molecular studies indicate that its mode-of-action is correlated with rapid loss of membrane integrity, depletion of free thiols/rapid ROS generation and inhibition of both the PKC and FLT3 signaling pathways. It should be noted that TDZD-8 induces cell-death effects not only on AML, but also chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) (25).

3-deazaneplanocin A (DZNep)

DZNep is a histone methyltransferase inhibitor that targets AML leukemia cells and shows certain capabilities on AML-stem/progenitor cells (33). It can disrupt polycomb-repressive complex 2 (PRC2), deplete enhancer of zeste homolog 2 (EZH2), reanimate TXNIP, inhibit thioredoxin activity, increase ROS, and then lead to AML cell apoptosis. A large body of knowledge has been accumulated for epigenetics-based treatment in AML. Compared to other epigenetic modulators, DZNep is unique in treating AML through a ROS-generation mechanism.

Mefloquine

Most recently, Sukhai et al. an antimalarial agent called ‘mefloquine’ for its capacity in targeting newly AML-stem cells (32). This study reveals a previously unappreciated mechanism for AML-stem cells killing. Mefloquine is able to disrupt lysosome integrity releasing hydrolases, lipases, proteases and cathepsins. This disruption subsequently increases the levels of ROS and triggers death of AML cells and stem cells in a caspase-independent manner. Mefloquine is widely used for malaria therapy and chemoprevention, its clinical safety has been already characterized in large cohorts of clinical trials. Its AML targeting efficacy will be next for evaluation.
Agents selectively induce AML-stem cells differentiation via ROS

Differentiation therapy achieves remarkable success in the acute promyelocytic leukemia (APL) therapy. Whether this therapeutic strategy can be extended to non-APL subtypes of AML, especially in targeting LSCs, still remains undefined. A recent study carried by Callens et al. suggests that inducing LSC differentiation by ROS-modulating agents is likely also be feasible (86,87). First, they find that iron deprivation therapy can induce generation of ROS in a time and dose-dependent manner. The iron deprivation is established using iron chelator deferasirox (DFX), deferoxamine (DFO), or transferrin receptor-specific antibody (e.g., TiR1 antibody and mAb A24). Second, leukemia cells (including primary AML cells from various subtypes) are promoted into mature monocytic differentiation. They also design paralleled experiments to reinforce the role of iron chelating treatments in ROS production, and to clarify the cause-effect between iron chelating treatments-induced ROS and monocytic differentiation of AML blast cells. Third, microarray analysis shows that iron chelating treatments-induced expression profiling is similar to that induced by Vitamin D3, a well-known inducer of HL60 cells monocytic differentiation. Last but not least, a refractory AML/MDS patient receiving oral DFX (1 g/d) and 25-hydroxycholecalciferol (4,000 IU/d) shows a decrease of AML blast cells and an increase of mature differentiated monocytes. Together, iron chelating—induced ROS generation and differentiation in AML, especially in refractory AML/MDS patients (likely due to the persistence of LSCs), opens up a possibility of using ROS-dependent LSC differentiation.

Concluding remarks and future challenges

The past 40 years have seen much progress in understanding biology and treatment of AML, as highlighted by, HSCs transplantation, sophisticated risk-stratification and great success of APL treatment. However, it seems to be hard for AML to be curable based on the current front-line therapies; they are limited by the inability in eradicating LSCs. Novel therapeutic regimens are required to target LSCs but not normal counterparts are required. The ROS-modulating agents are ideal because LSCs are much more susceptible to ROS than normal HSCs. In this review, we have given a relatively complete overview of agents that rely on ROS in part or in whole to potentially treat AML. Despite their promises, there are still many challenges that need to be addressed in the future:

(I) From our current understanding, both pro-oxidant (inducing oxidative stress) and antioxidant (reducing oxidative) environments are beneficial to cancer therapy. In terms of AML (especially AML stem cells), pro-oxidants seem to be much preferred over antioxidants. However, we are still ignorant of how to fine-tune balance between pro-oxidant and antioxidant to achieve the maximum therapeutic proficiency.

(II) We are aware of the fact that LSCs are more sensitive to ROS than normal HSCs, but do not know exactly why. There must be some built-in links between physiologic and pathophysiologic levels of ROS. It is critical for evaluating any ROS-generating agent and determining its optimal dosage in eradicating LSCs but sparing normal counterparts.

(III) ROS has multiple endogenous sources, and identifying these sources is always useful for designing efficient ROS-targeting agents. It is unclear which species of ROS is specific to leukemic cells (especially AML-stem cells). This clarification will greatly improve the efficiency of ROS-targeting AML therapy.

(IV) It should be clear that ROS is not the only mechanism of targeting LSCs. It is integrated into functional networks. Unfortunately, how ROS cooperates with other functional pathways to specially eradicating LSCs is little known.

(V) Increased levels of ROS can also promote cell proliferation, cell survival and drug resistance. It remains to test whether ROS-generation agents will induce secondary resistance.

(VI) The iron chelation therapy represents a new ROS-based strategy of inducing AML-stem cells differentiation. It is interesting to know whether this therapy is reproducible for sub-populations of AML stem cells.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.
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