Targeted therapies in the treatment of adult acute myeloid leukemias: current status and future perspectives

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Practice points

- This review dissects recent advancements in the molecular classification of acute myeloid leukemias (AMLs), which have led to the identification of pathogenic pathways that can be exploited with targeted agents and rational drug combinations. Furthermore, some reliable biomarkers have been identified, allowing both the identification of an AML subtype and the monitoring of the effect of the antileukemic treatment (i.e., *NPM1* mutant).
- The study of some molecular abnormalities occurring in AMLs has led to the identification of three AML subtypes bearing *NPM1*^{mut}, *FLT3-ITD* or *IDH1-2* mutations as suitable candidates to targeted therapies using specific inhibitors. Initial studies have shown encouraging results of FLT3 or IDH1-2 inhibitors in these AML subsets.
- This review considers the ongoing clinical studies based on the targeting of FLT3 and IDH1-2. These studies will define the ideal disease stage (i.e., at baseline or in complete remission patients after induction chemotherapy with MRD⁺) and possible drug combinations (i.e., FLT3 inhibitors in association with all-trans retinoic acid in *FLT3-ITD*-mutant patients).
- On the other hand, the targeting of membrane antigens CD33 and CD123, preferentially expressed on leukemic stem/progenitor cells, compared with the normal hematopoietic stem cells, represents an alternative strategy of leukemic cell targeting. Some molecules or antibodies targeting CD33 or CD123 are under clinical development with promising results.
- In conclusion, we believe that the development of efficient target therapies will consistently improve the standard of care of AMLs.

The rapid advancement of next-generation sequencing techniques and the identification of molecular driver events responsible for leukemia development are opening the door to new pharmacologic-targeted agents to tailor treatment of acute myeloid leukemia (AML) in individual patients. However, the use of targeted therapies in AML has met with only modest success. Molecular studies have identified AML subsets characterized by driver mutational events, such as *NPM1*, *FLT3-ITD* and *IDH1-2* mutations, and have provided preclinical evidence that the targeting of these mutant molecules could represent a valuable therapeutic strategy. Recent studies have provided the first pieces of evidence that FLT3 targeting in *FLT3*-mutant AMLs, IDH1/2 inhibition in *IDH*-mutant AMLs and targeting membrane molecules preferentially expressed on leukemic progenitor/stem cells, such as CD33 and CD123, represent a clinically valuable strategy.

KEYWORDS

- acute myeloid leukemia
- clinical trials
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- targeted therapy

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Acute myeloid leukemia (AML) is a genetically heterogeneous myeloid malignancy, predominantly occurring in adults and preferentially in older patients. AMLs need to be carefully classified according to their clinical and pathologic features and, particularly, require a morphologic, immunophenotypic, cytogenetic and molecular genetic analysis, allowing their classification in AML subtypes, associated with a different prognostic impact.

Various classifications of AMLs have been proposed in the time, starting from the original classification of AMLs by the French–American–British (FAB) group based on morphological and cytochemical criteria, followed by the WHO classification based on the identification of distinct clinic pathological entities, characterized according to cytogenetic or molecular genetic criteria. More recently, a widely adopted classification allows a prognostic stratification of AML patients [1].

This system proposed by the European Leukemia Network valid only in younger patients includes information about cytogenetics and the mutational status of *NPM1*, *FLT3* and *CEBPA* genes to define prognostic groups: low-risk, intermediate-risk and high-risk AMLs (Table 1) [1]. About 20–40% of adult AML patients fit in the low-risk group, 40–50% in the intermediate-risk group and 20–30% in the high-risk group.

A prognosis classification of AML solely based on molecular mutations and not on cytogenetics was proposed by Grossmann *et al.* [2]. According to this study, five distinct prognostic subgroups were identified: firstly, very favorable: *PML-RARA* re-arrangement or *CEBPA* double mutations (overall survival [OS] at 3 years: 82.9%); secondly, favorable: *RUNX1-RUNX1T1*, *CFFB-MYH11* or *NPM1* mutation without Fms-like tyrosine kinase–internal tandem duplication (*FLT3*–ITD; OS at 3 years: 62.6%); thirdly, intermediate: none of the mutations leading to assignment into groups 1, 2, 4 or 5 (OS at 3 years: 44.2%); fourthly, unfavorable: *MLL-PTD* and/or *RUNX1* mutation and/or *ASXL1* mutation (OS at 3 years: 21.9%) and fifthly, very unfavorable: *TP53* mutation (OS at 3 years: 0%). This comprehensive molecular characterization seems to provide a more powerful model for prognostication than cytogenetics [2].

The development of genome-sequencing techniques allowed to define the spectrum of gene mutations present in the various AML subtypes and to study how these mutations can evolve in the natural history of disease or following relapse. These studies have defined the most recurrent mutations observed in AMLs, relevant for pathogenesis, including transcription factor fusion (15–20% of cases), the *NPM1* gene (25–30%

Table 1. Prognostic classification of acute myeloid leukemias according to the European Leukemia Network.			
Prognostic group	AML subtypes	Probability of CR (%)	Probability of relapse (%)
Low risk	t(8;21)(q22;q22) inv(16)(p13.1;q22) t(16;16)(p13.1;q22) t(15;17)(q22;q12) NK and NPM1 ⁺ /FLT3–ITD [–] NK and CEBPA ^{+/+}	80–95	5–40
Intermediate risk	NK and NPM1 ⁺ /FLT3–ITD [–] NK and NPM1 ⁺ /FLT3–ITD ⁺ NK and NPM1 [–] /FLT3–ITD ⁺ t(9;11) Cytogenetic abnormalities not in the low- or high-risk groups t(8;21), inv(16) and t(16;16) with KIT mutations	50–80	50–80
High risk	t(6;9), t(3;3), inv(3) Monosomy 7 (–7) Monosomy 5 (–5) Deletion of long arm (q) of chromosome 7 (–7q) Abnormalities of 3q, 17p, 11q Multiple cytogenetic abnormalities (≥3–5)	<50	>90
AML: Acute myeloid leukemia; CR: Complete remission; NK: Normal karyotype.			

of cases), tumor-suppressor genes (such as *TP53* and *WT1*, 15–18% of cases), DNA-methylation-related genes (such as *DNMT3A*, *IDH1*, *IDH2*, *TET2*, 40–50% of cases), signaling genes (such as *FLT3*, *KIT*, *NRAS*, *KRAS*, 55–60% of cases), chromatin-modifying genes (such as *ASXL1*, *MLL-PTD*, *MLL* fusions, *RDM6A*, about 30% of cases), myeloid transcription factor genes (such as *RUNX1*, *CEBPA*, 20–25% of cases), cohesion complex (such as cohesin, about 13%) and spliceosome complex genes (about 14%) [3].

More recently, Papaemmanuil *et al.* have proposed a genomic classification that identifies 13 AML subtypes, not overlapping, reported in **Figure 1** [4].

Another recent study based on the genomic analysis of a large set of patients (664 adult AML patients) analyzed the spectrum and the prognostic relevance of the main driver gene mutations. Following the results of this large screening, it was proposed a subdivision in nine nonoverlapping groups, allowing the classification of 78% of all patients: CBFAML: *RUNX1–RUNX1T1*, *CBFB–MYH11* (10%); *KTM2A* (*MLL*)-rearranged (6%); *GATA2*, *MECOM* (2%); *DEK–NUP214* (1%); *CEBPA* double mutated (4%); *TP53*-mutated (10%); *NPM1*-mutated (33%); *RUNX1*-mutated (15%) [5]. *TP53*-mutated and *RUNX1*-mutated AMLs, both associated with a poor outcome, are clearly more frequent among older (>60 years) than younger (<60 years) AMLs [5]. *TP53* mutations are very frequent among patients with adverse cytogenetic profile [5]. The presence of *DNMT3A* mutations (predominantly observed in patients of intermediate risk) associated with inferior overall survival among younger, but not older AML patients; this phenomenon was particularly evident when these mutations associated with *NPM1* and *FLT3–ITD* mutations [5]. A median number of four driver mutations per patient was observed, changing in various AML groups [5].

Therefore, it is not surprising that several of the molecularly identified AML subtypes were now included in the new 2016 WHO classification of AMLs, including AML with mutated *NPM1*, AML with biallelic mutations of *CEBPA*, AML with mutated *RUNX1* and AML with *KMT2A* (*MLL*) rearranged [6].

It is of interest to note that the initial classification of AMLs was entirely based on cytomorphological subtypes defined according to the FAB criteria [7,8]. This classification has played a very important role on the laboratory

and clinical studies on AML and, after its proposal, attempts have been made to define correspondences between specific molecular abnormalities and FAB subtypes. Thus, AML with t(15;17)/PML-RARA is observed in FAB M3 subtype; AML with *RUNX1–RUNX1T1* fusion in FABM2/M1; AML with *inv(16)/CBFB–MYH11* with abnormal eosinophil in FABM4 eo; 11q23/*MLL* re-arrangements in FABM5a/M5b; AML with *NPM1–MLF1* fusion in FAB M6. Recently, Rose *et al.* have performed the analysis of the correspondence between the most common gene mutations observed in AML, and their FAB subtype [9]. This analysis provided evidence that the spectrum of gene mutations observed in every FAB subtype is very wide. However, some gene mutations display a preferential association with FAB subtypes: *RUNX1* and *ASXL1* mutations are most frequent in FAB M0 AMLs; *TP53* mutations are preferentially observed in FABM6 AMLs; *NPM1* mutations are most frequent in M5b, M4 and M5a AMLs; *DNMT3A* mutations are more frequently associated with M5b and M4 AMLs; *IDH1/IDH2* mutations are preferentially observed in FAB M0, M1 and M2 AMLs; *FLT3–ITD* mutations are preferentially observed in FAB M1, M4 and M5b AMLs; *CEBPA* mutations (monoallelic or biallelic) are preferentially observed in FAB M1 and M2 AMLs; *NRAS* mutations are preferentially observed in FAB M4, M5a and M5b [9].

The study of multiple genetic alterations occurring in AMLs was of fundamental importance not only for the understanding of the pathogenesis of this disease but also for the understanding of the clonal architecture, dynamic and evolution during the natural history of the disease. To explain the presence of multiple driver mutations in the leukemic cells of each AML patient, a model of leukemia development was proposed: in this model, the various AML mutations are sequentially acquired in successive clones of hematopoietic stem cells (HSCs), unless the mutations confer self-renewal potential on a more differentiated cell [10].

Several recent studies have provided support to this model. Particularly, the study of few AML patients with *FLT3* mutations showed that multiple genetic alterations serially accumulate at the level of HSCs, but a part of these HSCs display only a part of these mutations and are functionally normal in that they are able to originate multilineage hematopoiesis, like normal HSCs; these HSCs are defined as pre-leukemic

Figure 1. Molecular classification of acute myeloid leukemias according to the study of Papaemmanuil *et al.* This classification was based on the results derived from the whole-genome sequencing of 1540 adult AMLs [4]. This analysis allowed to classify AMLs in 13 nonoverlapping molecular subtypes, characterized by a typical pattern of driver mutations: AML with *NPM1* mutation, corresponding to about 27% of all AMLs (these AMLs frequently display *DNMT3A*, *FLT3-ITD*, *NRAS*, *TET2* and *PTPN11* mutations, in decreasing order); AML with mutated chromatin, RNA splicing genes such as *RUNX1*, *MLL-PTD*, *ASXL1* and *STAG2*, corresponding to about 18% of all AMLs (these AMLs frequently display *DNMT3A*, *NRAS*, *TET2* and *FLT3-ITD* mutations); AML with mutated chromatin, chromosomal and aneuploidy or both, including complex karyotype, -5/5q, -7/7q, *TP53* mutations, -17/17p and -12/12p, corresponding to about 13% of all AMLs; AML with *inv(16)* (p13.1q22) or *t(16;16)* (p13.1; q22); *CBFB-MYH11*, corresponding to about 5% of all AMLs (these AMLs frequently display *NRAS*, *KIT* and *FLT3-ITD* mutations); AML with biallelic *CEBPA* mutations, corresponding to about 4% of all AMLs (these AMLs frequently exhibit *NRAS*, *WT1* and *GATA2* mutations); AML with *t(15;17)* corresponding to about 4% of all AMLs (these AMLs frequently exhibit *FLT3-ITD* and *WT1* mutations); AML with *t(8;21)* (q 22; q 22); *RUNX1-RUNX1T1*, corresponding to about 4% of all AMLs (these AMLs frequently display *KIT* mutations, -Y and -9q); AML with *MLL* fusion genes; *t(x;11)* (x;q23), corresponding to about 3% of all AMLs (these AMLs frequently exhibit *NRAS* mutations); AML with *inv(3)* (q21q26.2) or *t(3;3)* (q 21; q26.2) ; *GATA2*, *MECOM* (*EVI1*), corresponding to about 1% of all AMLs (these AMLs frequently display -7 and *KRAS*, *NRAS*, *PTPN11*, *ETV6*, *PHF66* and *SF3B1* mutations), AML with *IDH-R172* mutations, in the absence of other class-defining alterations, corresponding to about 1% of all AMLs (these AMLs frequently exhibit *DNMT3A* mutations and +8/8q); AML with *t(6;9)* (p23;q34); *DEK-NUP214*, corresponding to about 1% of all AMLs (these AMLs frequently display *FLT3-ITD* and *KRAS* mutations); AML with driver mutations, but no detected class-defining alteration (about 11% of all AMLs); AML with no detected driver mutation (about 4% of all AMLs); AML that meets criteria for ≥2 genomic subgroups (about 4% of all AMLs) [4]. These molecularly defined AML groups are prognostically relevant in that: among gene fusion groups, *inv(16)* and *t(15;17)* are associated with a good prognosis, *t(8;21)* with an intermediate prognosis and *t(6;9)*, *MLL* fusion and *inv(3)* with a poor outcome. Among the AMLs with no gene fusions, *CEBPA* biallelic and *IDH2-R172* had the best outcome, *NPM1*-mutated and intermediate risk and chromatin-spliceosome and *TP53*-aneuploidy a poor risk [4]. The outcome of *NPM1*-mutant AMLs was related to the presence of the concomitant *DNMT3A* and *FLT3-ITD* mutations: thus, the *NPM1/DNMT3A*, *FLT3-ITD* mutant subgroup had a clearly poorer survival than *NPM1/DNMT3A*-mutant AMLs [4].

AML: Acute myeloid leukemia.

Data taken from [4].

stem cells (pre-LSCs) and are considered the precursors of the cells that initiate and maintain the leukemic process [11]. The mutations observed in these pre-LSCs occur at the level of genes like *TET2*, *SMC1A*, *CTCF*, but not of *FLT3*, thus implying that *FLT3* mutations are acquired at later times during the evolution of the leukemic process (Figure 2) [11].

Other studies have supported the finding that mutations occurring in pre-LSCs are different

from those present in the bulk tumor: thus, while early mutations present in pre-LSCs frequently involve genes related to epigenetic control or acting as chromatin remodeling regulators, driver mutations at the level of myeloid transcription factors and signal transduction molecules occur later and are not present in pre-LSCs (Figure 2) [12].

Furthermore, Wong *et al.* described the presence of chemotherapy-resistant HSCs in

Figure 2. Clonal mutational evolution of acute myeloid leukemia. The picture shows the sequential acquisition of somatic mutations occurring during leukemia development from a preleukemic condition until to leukemia relapse. Three different patterns of leukemic evolution are observed: founding mutations are enriched in epigenetic regulatory genes (*DNMT3A*, *TET2*, *IDH1*, *IDH2*, *ASXL1*), while late mutations occur preferentially at the level of genes involved in proliferative activated signaling (*FLT3*, *KRAS/NRAS*, *NPM1*); in other AMLs, founding mutations are represented by *CBF* and *MLL* rearrangements, which induce both epigenetic and transcription factor deregulation, and in these leukemias there is less requirement for additional genetic lesions; in other AMLs, founding mutations occur at the level of *TP53* alone or together with an epigenetic gene (*DNMT3A*) and in these leukemia late events are represented by complex karyotype lesions and, less frequently, by *NPM1*, *RAS* and *FLT3* mutations.

HSC: Hematopoietic stem cell.

AML patients after chemotherapy: these HSCs appeared to expand rapidly after depletion of the bulk leukemic cell population by chemotherapy [13]. These clones harbored mutations at the level of epigenetic regulators and seemingly represent pre-LSCs, primed to leukemic transformation, capable of expansion to repopulate hematopoiesis [13].

A recent study analyzed the mutational clonal evolution in a large set of AML patients, reaching the conclusion that not only mutational events at the level of epigenetic regulator genes are observed in pre-LSCs but also mutations in *CBF* or *MLL* translocations or *TP53* mutations may represent early mutational events observed in pre-LSCs [14]. According to these findings, the authors of this study have delineated various patterns of AML genetic evolution: first, a typical pattern of evolution involves early lesions in *DNMT3A*, *TET2* and *ASXL1*, aggregating together or with other mutations in epigenetic regulators, followed by mutations at the level of *NPM1* or in hematopoietic transcription factors and then by mutations in genes involved in signaling pathways, such as *FLT3* and *RAS*; second, an alternative pathway of leukemic transformation, where *CBF* and *MLL* rearrangements induce both hematopoietic transcription factor and epigenetic deregulation, and therefore require less additional genetic lesion for leukemia development; third, another pathway in which *TP53* mutation is the initiation, preleukemic event, followed by mutations of epigenetic regulators and then by complex cytogenetic abnormalities [14].

The identification of pre-LSC clones in AMLs has led to hypothesize the existence of similar clones in the blood of normal individuals and, particularly, of old subjects because it is very well known that age favors mutation accumulation. In line with this hypothesis, while benign clones bearing mutations such as *DNMT3A* and/or *TET2* are rarely observed in subjects less than 60-year-old, they were detected in 10–20% of individuals with an age of more than 70 years [15,16]. Hematopoietic clones identified in these old subjects were called ‘clonal hematopoiesis of indetermined potential’ and their presence is associated with an increased risk of developing malignancy [17].

However, these studies could detect only common hematopoietic clones – that is, with greater than 0.02 variant allele fraction, due to the error rate of next-generation sequencing. The development of new methods to identify these mutations

below this threshold, allowed to demonstrate that clonal hematopoiesis is observed in 99% of old subjects [18]; often these mutations are stable longitudinally and are observed in multiple lineages, thus implying their origin at the level of long-lived HSCs and HPCs [18].

The actual standard paradigm of AML treatment

The standard AML treatment involves first the induction chemotherapy followed by consolidation or intensification treatment. The main aim of the induction chemotherapy is to induce a disease remission and to eliminate as much as possible leukemic cells. The induction treatments usually involve a combination chemotherapy based on the administration of cytarabine (100–200 mg/m²) for 7 days and an anthracycline (daunorubicin or idarubicin) for 3 days. In the eventuality of persistence of leukemic cells after this induction cycle, the AML patients are treated with the same antineoplastic agents, again following a 3 + 7 schedule. In these induction/intensification treatments, the dosage of the various drugs may change either in the induction (standard or high cytarabine doses) or during the intensification (low, middle or high daunorubicin doses); however, it is unclear whether the high-dose regimens yield better results than standard-dose regimens [19,20].

Thus, a recent study provided evidence that increasing intensity of therapies at diagnosis does not improve survival of adult patients with AML [21]. Using these intensive remission induction chemotherapy regimens, 60–80% of younger AML patients (<60 years) and 40–60% of older AML patients achieve a complete response. Given the greater treatment-related morbidity and mortality observed in older AML patients, a significant proportion of them is not eligible for intensive remission induction and are usually considered for single-agent therapies with lower toxicity profiles [22].

There is increasing evidence that the use of hypomethylating agents (such as azacitidine or decitabine) resulted in a better outcome in these older AML patients than current care treatments, including intensive chemotherapy. The meta-analysis of the randomized controlled trials showed that azacitidine treatments resulted in an improvement of overall survival compared with standard care [23]. However, it is still unclear whether hypomethylating agents are a superior alternative to intensive chemotherapy

for older AML patients who can tolerate these treatments.

Encouraging results are also observed with new hypomethylating agents, such as guadecitabine [24] or with sequential regimens involving azacytidine and lenalidomide administration [25].

The dilemma of best postremission therapy is still unresolved mainly due to the paucity of randomized controlled studies. Post-remission treatment of AMLs may consist of continuing chemotherapy or transplantation using either autologous or allogeneic stem cells. A patient's leukemic genetic risk profile, predicting the risk of relapsing and other factors of clinical risk are usually used for the choice of the optimal postremission treatment (Table 1). Usually, patients with favorable subtypes of AML receive chemotherapeutic consolidation, while allogeneic hematopoietic stem cells transplantation (allo-HSCT) is the preferred type of postremission therapy in poor-risk AMLs [26]. The choice of postremission optimal therapy in intermediate-risk AMLs is more debated and, in these patients, the place of allo-HSCT or autologous HSCT (auto-HSCT) limited to patients without minimal residual disease is still undefined [26].

Recent studies have shown that in AML patients aged 40–60 years, allo-HSCT is a treatment preferred over chemotherapy as postremission treatment in the groups associated with intermediate and poor risk, whereas auto-HSCT remains a valuable treatment option in patients with intermediate-risk AML, without MRD [27]. Auto-HSCT is usually associated with prolonged disease-free survival compared with chemotherapy, particularly in low-risk and intermediate-risk patients; however, randomized clinical trials are strictly required to assess the real impact of auto-HSCT, compared with consolidation chemotherapy [28].

Another study evaluated the optimal postremission treatment in cytogenetically normal AML subclassified on the basis of *NPM1* and *FLT3*–ITD allelic ratio: a favorable OS was observed for patients with mutated *NPM1* without *FLT3*–ITD, while patients with a high *FLT3*–ITD ratio displayed poor OS; in AML patients with mutated *NPM1*, without *FLT3*–ITD or with *FLT3*–ITD at low allelic burden, allo-HSCT resulted in better OS and relapse-free survival (RFS) compared with chemotherapy or auto-HSCT [29].

The choice of optimal postremission therapy in older/elderly AML patients is poorly defined.

AML patients with a favorable-risk assessment should receive intermediate-dose cytarabine-based regimens, whereas in patients with intermediate-risk or adverse-risk AML allo-HSCT is associated with a better OS than chemotherapy, auto-HST or no postremission therapy [30].

Allo-HSCT is a potentially curative approach in patients with AML. To expand this therapeutic strategy to elderly and medically infirm patients not eligible for standard myeloablative conditioning, a reduced-intensity conditioning was widely introduced over the past 15 years. Several studies have shown similar survival of AML patients after HSCT with myeloablative conditioning or reduced-intensity conditioning and recent studies have shown that this survival remains similar also on long-term outcome (beyond 10 years) [31].

Recent studies have highlighted the importance of detection of MRD at the remission stage for both its prognostic implications and for its possible role for treatment selection [32].

However, clinical choices based on MRD detection are extremely challenging because they depend on the availability of a disease biomarkers specific of the leukemic disease and present at the level of all the leukemic cell population; the ideal marker is a leukemia-specific molecular marker (i.e., mutant *NPM1* or *RUNX1/RUNX1T1*) whose detection must be based on a validated sensitive and quantitative assay (i.e., quantitative real-time reverse transcription PCR) after determination of appropriate cut-off levels [33]. The positivity of these molecular markers is predictive of disease relapse [34].

In this context, the results of a recent study were particularly informative. This study was based on the analysis of a large set of AML patients with normal karyotype and with *NPM1* mutations. In this group of AML patients, the presence of *FLT3*–ITD and mutated *DNMT3A* had a significant effect on the outcome; importantly, on multivariate analysis, the presence of minimal residual disease – that is, the persistence of mutated *NPM1* transcripts, in the peripheral blood of patients is a highly prognostic factor of disease relapse and outcome [35]. Furthermore, the detection of a mutated *NPM1* was shown to be a very reliable and stable marker of disease relapse in that it was clearly positive in 69 out of 70 AML patients exhibiting disease relapse [35]. These observations strongly support the importance of detection of MRD to assess response and to identify a group of patients

with poor prognosis who may be candidate for transplantation and/or for new therapies [35].

From 20 to 30% of AML patients are refractory to the remission induction chemotherapy; furthermore, disease relapse occurs in a significant proportion of AML patients within 3 years after the initial diagnosis. The treatment of these refractory/relapsing AML patients is a very challenging problem and there is no indication of an optimal salvage regimen; the goal of this salvage therapy is to bridge the patients to all-HSCT, the only treatment with some perspectives of a curative effect in these patients. In 30–50% of cases, the salvage therapy induces a CR [36].

Refractory/relapsed patients not amenable to allo-HSCT should be enrolled in clinical trials based on novel therapies.

Factors limiting the development of a targeted therapy for AML

In a perspective review paper published on *Blood* in 2015, Estey *et al.* analyzed the main difficulties that hampered the testing and the clinical success of targeted therapies in AMLs [37]. The main difficulties were believed to derive from the limitations of preclinical models in capturing inter- and inpatients' genomic heterogeneity and of clinical trials usually based on the use of a single-agent clinical approach during early drug development [37]. Thus, both limiting factors do not take into the appropriate account the genomic complexity of AMLs (more than one driver mutation is present in many AMLs), the clonal architecture of the leukemia (the therapy target is not expressed in all leukemic clones) and the molecular heterogeneity of the target (the mutant target is heterogeneously mutated and the target agent is active only against the main mutant) [37].

Thus, the use of *in vitro* and *in vivo* models using primary leukemic cells is required, together with the development of more robust animal preclinical models involving the use of organoids and combinations of genetic and pharmacologic approaches [37].

A recent study very well clarifies all the difficulties related to the development of a preclinical model of AMLs suitable for therapeutic studies. Thus, Wang *et al.* have developed patient-derived xenotransplants (PDX) from a large set of AML patients and showed that, based on variant allele frequency changes, 50% of patient tumor–PDX pairs are concordant, while the remaining 50% is at some extent, discordant [38]. Therefore, genomic profiling of PDX and corresponding

patient samples is strictly required to ensure concordance before performing mechanistic or therapeutic studies [38].

The current clinical trials with new agents are biased by the inclusion of only relapsed refractory, or unfit newly diagnosed patients [37]. Therefore, an initial selection of patients limited to those with a specific genetic aberration targeted by the new drug, as well as the reduction of the times existing between the first use of these targeted drugs and their use in combination with chemotherapy should considerably contribute to reduce the times of clinical development of these new agents [38].

In spite of all these limitations and problems, the development of targeted therapies is strictly required to attempt to improve the current outcome of AMLs. The choice of new strategies allowed in the last years the initial development in AMLs of successful clinical trials based on targeted therapy and supported the idea that some AML subsets must be treated with a dedicated, molecular-targeted therapy [39].

Two very successful examples of targeted therapy are the therapy with all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) for acute promyelocytic leukemia and the therapy of Philadelphia chromosome-positive chronic myeloid leukemia (CML) with BCR-ABL tyrosine kinase inhibitors (TKIs). Particularly, ATRA and ATO, by targeting the PML-RAR α fusion protein, allowed to improve the survival rates of APL patients up to 90%, with a limited treatment-related toxicity [40].

First-generation and second-generation BCR-ABL TKIs have dramatically changed the natural history of CML and represent an important success of targeted therapy [40]. However, some patients nonetheless demonstrate primary or secondary resistance to such therapy and require an alternative therapeutic strategy [41].

BCR-ABL TKIs fail in the large majority of patients to completely eradicate quiescent CML LSCs. However, several recent reports show preliminary evidence about pharmacologic strategies suitable to achieve the eradication of these cells: the glitazones, antidiabetic drugs that are agonists of PPAR γ are able to purge the residual CML LSC pool present in patients undergoing therapy with BCR-ABL TKIs [42]; combined targeting of BCL-2 and BCR-ABL eradicates CML stem cells [43]; dual targeting of p53 and c-MYC selectively eliminates CML stem cells, while sparing normal HSCs [44].

In this review, we analyze recent studies supporting at therapeutic level the targeting of some molecules either mutated (NPM1, FLT3 and IDH1–2) or overexpressed (CD33 and CD123) in AMLs. The main properties of these molecules, their abnormalities in leukemic cells and their pharmacologic targeting are summarized in **Table 2**.

AML with NPM1 mutation

NPM1 exon 12 mutations have been reported to be involved in leukemogenesis and are detected in about 30–40% of AML cases. NPM1 mutations result in the cytoplasmic dislocation of NPM1. The outcome of NPM1-mutated AML treatment is influenced by the presence or absence of cooperating FLT3–ITD and DNMT3A mutations. Particularly, about 40–50% have FLT3–ITD, about 50% DNMT3A (a part of these patients are comutated for both FLT3–ITD and DNMT3A); in addition, about 10% of these patients display IDH1 mutation, 15% IDH2 mutations, 20% NRAS mutation and 24% FLT3 point mutations [35]. Patients with mutated NPM1, but not co-existing FLT3–ITD and DNMT3A mutations have a better prognosis than those displaying these two co-existing mutations. Thus, patients with mutated NPM1 and co-existing FLT3–ITD or DNMT3A mutations (corresponding to about 60–70% of NPM1-mutated AML) have a poorer prognosis and may be considered as candidates for transplantation [35].

Therefore, the treatment of NPM1-mutated AMLs is a challenging problem due to their heterogeneity. At the level of therapy, these NPM1-mutated AMLs could be treated either attempting a direct targeting of the mutated NPM1 or of the mutated co-existing genes, such as FLT3–ITD. Concerning the NPM1 targeting, two strategies have been developed through the identification of agents that inhibit NPM1 function and of compounds that induce NPM1 degradation. The first type of molecule is represented by deguelin, a selective silencer of the NPM1 mutant that stimulates apoptosis and induces differentiation in AML cells carrying the NPM1 mutation [45].

The second type of molecules is represented by ATRA and ATO, two molecules used with great success in the treatment of APL. These studies were originated from an initial observation showing that inhibition of NPM1 expression sensitizes mutant NPM1 AML cells to ATRA [46].

Two studies have simultaneously reported that exposure of NPM1-mutant leukemic cells to ATO and ATRA induces the selective proteasome-mediated degradation of the mutant NPM1 protein, accompanied by nucleolar redistribution of the wild-type NPM1 protein, induction of apoptosis and/or differentiation [47,48]. Importantly, these biochemical effects were selectively observed only in NPM1-mutant AML cells, including primary leukemic blasts and were dependent upon induction of oxidative stress [48].

Since ATO and ATRA are in current clinical use, these findings offer a unique opportunity for clinical translations. However, the eventual successful development will require the identification of the optimal patient target population (i.e., all NPM1-mutant AMLs or only those FLT3-WT and DNMT3A-WT?) and of the disease stage to be treated (i.e., MRD after remission induction therapy or relapsing disease?).

A recent study showed an original strategy to target NPM1-mutant AMLs. This strategy was based on the analysis of the mechanisms through which NPM1^{mut} cells maintain aberrant gene expression. Particularly, it was shown that NPM1^{mut}-driven leukemogenesis requires both HOX and MEIS1 expression, controlled by specific chromatin-regulatory complexes by a process involving menin–MLL1 interaction and the activity of the H3K79 methyltransferase DOT1L [49]. Interestingly, small-molecule inhibitors of menin–MLL1 and DOT1L resulted in inhibition of leukemic growth and induction of leukemic cell differentiation [49]. Interestingly, the treatment with these two inhibitors resulted also in a marked inhibition of FLT3 expression [49].

FLT3 targeting

FLT3 is a tyrosine kinase receptor playing a key role in the control of proliferation and differentiation of HSCs and of some hematopoietic lineages. The *FLT3* gene is frequently mutated in AMLs: a constitutive activation of FLT3 receptor by ITD is an event observed in 20–30% of AMLs; in about 5–8% of AML patients is observed a mutation of FLT3 at the level of the activation loop of the tyrosine kinase domain (FLT3/TKD mutations). FLT3–ITD mutations lead to ligand independent, autophosphorylation and constitutive activation of FLT3 receptor and its downstream signaling effectors, such as PI3K/AKT, RAS/RAF/MEK

Table 2. Molecules targeted for the development of new anti-acute myeloid leukemia therapy, their abnormalities in leukemic cells, their pharmacologic targeting and ongoing clinical trials.

Biochemical target	AML-related alteration	Mode of action	Target 'druggable'	Drugs
NPM1	Mutated in 30–40% of AMLs	Nucleolar component	Compounds inducing NPM1 degradation: – ATRA – ATO Compound inhibiting NPM1-mediated gene expression: – Menin-MLL1 – inhibitors – DTO1L inhibitors	ATRA and ATO MI-503 (inhibitor of menin-MLL1) and EPZ4777 (DTO1L inhibitor)
IDH1 and IDH2	Mutated in about 10% AMLs	Conversion of isocitrate to α -keto-glutarate Mutant enzymes acquire a neomorphic function: isocitrate is converted to R2-hydroxyglutarate	Small-molecule inhibitors (AG-120: inhibitor of IDH1 enzyme AG-221: inhibitor of IDH2 enzyme)	AG-120 (NCT 02074839) AG-221 (NCT 01915498, NCT 02577406)
FLT3	FLT3-ITD: about 25% AMLs FLT3-TKD: about 10% AMLs	Receptor tyrosine kinase for FLT3 ligand Mutant receptors display constitutive receptor activation	Small-molecule multikinase inhibitors with activity against mutant FLT3 in AMLs: sorafenib, midostaurin, quizartinib, crenolanib	Sorafenib (NCT02196857, NCT01253070) Midostaurin (NCT00651261) Quizartinib (NCT01892371, NCT02039726) Crenolanib (NCT016557682, NCT02400281, NCT02283177)
CD33	Increased CD33 expression on leukemic blasts compared with normal bone marrow myeloid precursors	Member of the SIGLECS Myeloid differentiation antigen not expressed on HSCs	Specific mAbs. SGN-33A: mAb drug conjugated directed at CD33 AMG-330: monoclonal bispecific antibody directed at CD33 and CD3.	SGN-33A (NCT02326584, NCT019002329) AMG-330 (NCT02520427)
CD123 (IL-3R alpha chain)	CD123 is overexpressed on AML leukemic blasts, compared with BM myeloid precursors. CD123 is expressed on leukemic stem cells, but not on normal HSCs	High-affinity receptor for IL-3. The activated IL-3 plays multiple biological functions, being involved in the control of normal and malignant hemopoiesis, native and adaptive immunity and inflammatory response	Specific mAbs or IL-3 ligand SL401: recombinant human IL-3 fused to truncated diphtheria toxin Talacotuzumab (CSL362): mAb to human IL-3R alpha chain IMGN362: anti-CD123 drug-conjugate	SL 401 (NCT02113982) Talacotuzumab (NCT02472145, NCT01632852)

AML: Acute myeloid leukemia; ATO: Arsenic trioxide; ATRA: All-trans retinoic acid; BM: Bone marrow; FLT3: Fms-like tyrosine kinase; HSC: Hematopoietic stem cell; ITD: Internal tandem duplication; mAb: Monoclonal antibody; SIGLECS: Sialic acid-binding immunoglobulin-like lectins; TKD: Tyrosine kinase domain.

and JAK/STAT5 pathways, which collectively lead to cellular proliferation (growth advantage) and immortalization; the presence of FLT3–ITD mutations is associated with poor clinical outcomes and an increased relapse rate, while FLT3/TKD mutations do not have a major clinical impact.

Given the high frequency of FLT3–ITD mutations in AMLs and the negative prognosis of these leukemias, a great effort was performed to isolate small molecules acting as potent FLT3 inhibitors. Many FLT3 inhibitors were isolated and characterized during the last years and pertain to: first, the first generation of FLT3 inhibitors

including midostaurin, sorafenib, sunitinib and lestaurtinib, characterized by a relative wide spectrum of TKI activity, not being specific for FLT3; second, the second generation of FLT3 inhibitors, including quizartinib, gilteritinib, crenolanib and ponatinib, characterized by a major selectivity and potency for mutant FLT3 (Table 3). For a detailed analysis of the properties of all these FLT3 inhibitors, the readers are referred to some excellent recent reviews [50].

It is important to point out that the clinical impact of FLT3 kinase inhibitors has been limited; resistant clones have emerged rapidly and this problem has been only partially overcome

Table 3. First-generation and second-generation Fms-like tyrosine 3 inhibitors under clinical investigation in acute myeloid leukemia patients.

Inhibitor	Structure	Target	IC ₅₀ against FLT3-ITD (nM)	Trial phase	Company
Lestaurtinib (CEP-701)		JAK2, FLT3, TrK A	3	III	Cephalon
Midostaurin (PKC 412)		FLT3, c-KIT, PDGFRB, VEGFR	<10	II/III	Novartis
Sunitinib (Sutent)		FLT3, c-KIT, KDR, PDGFR	4	I/II	Pfizer
Tandutinib (MLN 518)		FLT3, PDGFR, c-KIT	220	I	Millenium Pharmaceuticals
Crenolanib (CP-868-596)		FLT3, PDGFR	1-2	II/III	Arog Pharmaceuticals
Gilteritinib (ASPO 2215)		FLT3, AXL	0.29	III	Astellas Pharma
Ponatinib (AP 24 534, Iclusig)		BCR/ABL, FLT3, c-KIT, FGFR1, PDGFRA	4	I/II	ARIAD Pharmaceuticals

Table 3. First-generation and second-generation Fms-like tyrosine 3 inhibitors under clinical investigation in acute myeloid leukemia patients (cont.).

Inhibitor	Structure	Target	IC ₅₀ against FLT3-ITD (nM)	Trial phase	Company
Quizartinib (AC 220)		FLT3, c-KIT, PDGFRA	1.1	II	AMBIT Biosciences
Sorafenib (Nexavar)		FLT3, c-KIT, VEGFR, PDGFR, RAF-1	2	III	Bayer and Onyx Pharmaceuticals

by second-generation FLT3 inhibitors [50]. Furthermore, FLT3-ITD displays some instability during leukemia progression; in fact, comparative mutation analysis of a large set of primary and relapsed paired FLT3-ITD-mutated AML samples showed high stability of mutations in DNMT3A, NPM1, IDH2, RUNX1 and TET2, whereas less stability was observed in FLT3-ITD (in fact, in a minority of patients FLT3-ITD mutation was lost at relapse [51]).

In the present review are analyzed only the FLT3 inhibitors at more advanced stage of clinical development.

Midostaurin is the FLT3 inhibitor in the most advanced stage of clinical development. This inhibitor, active against both FLT3-ITD and FLT3-TKD mutants, was investigated in several Phase I/II clinical trials providing initial evidence that reduced leukemic blasts in FLT3-mutant AMLs. The results of these initial studies were analyzed in detail in a recent review paper [52].

The preliminary results of the randomized Phase III clinical trial RATIFY (NCT00651261) are promising: clinical benefit in younger/AML patients treated with Midostaurin in association to standard induction chemotherapy [53]. In this study, 717 patients were randomized to receive standard remission induction and consolidation chemotherapy either without or with Midostaurin [53]. Importantly, patients in the Midostaurin arm had a 23% improvement at the level of overall survival [53]. Furthermore, 57% of the patients enrolled in the RATIFY trial undergo allogenic stem cell transplantation:

event-free survival was 3.0 months in the Midostaurin arm [53]. However, an important limitation of this study is related to the use of FLT3 inhibitor in induction, consolidation and maintenance phases and is unclear at which phase the drug administration improves the antileukemic response. Midostaurin is being evaluated in association with other antileukemic drugs, such as hypomethylating agents or in post-transplantation maintenance. Potentially interesting is the second study aiming to evaluate the capacity of Midostaurin to prevent leukemia relapse, when administered within 60 days to FLT3-ITD AML patients undergoing allogeneic HSCT (NCT01883362). The main objective of this study is to evaluate the effect of Midostaurin on relapse-free survival.

Among the various second-generation FLT3 inhibitors particularly promising are results obtained with gilteritinib (see Table 3). This compound is a potent inhibitor of both FLT3-ITD and FLT3-TKD. The results obtained in a Phase I/II (CHRYSLIS trial, NCT02014558) were recently reported, showing in 82 refractory or relapsing FLT3-mutated AMLs an overall response rate of 57%; the overall response rate was higher (63%) in patients treated with an ≥80 mg dose [54].

A Phase III study (NCT 02421939) is ongoing comparing gilteritinib + chemotherapy with chemotherapy + placebo.

In the future, FLT3 inhibitors may be used alongside conventional chemotherapy in induction regimens, as a maintenance therapy, or in relapsed/refractory patients as bridge to

transplantation; additional advanced clinical trials will be strictly required to explore and define these possibilities.

Targeting of IDH-mutant AMLs

IDH1 and IDH2 are key metabolic enzymes responsible for the enzymatic conversion of isocitrate to α -ketoglutarate. IDH1/2 mutations occur in about 20% of AML patients: 6–16% IDH1 mutations; 8–19% IDH2-mutated. IDH-mutated AMLs are characterized by a preferential occurrence in older patients, an increased percentage of leukemic blasts in the bone marrow and peripheral blood, a more frequent association with NPM1 and FLT3 mutations, a frequent association with DNMT3A mutations and mutual exclusivity with TET2 and WT1 mutation [55].

Mutation of IDH1 or IDH2 enzymes at the level of enzyme active sites results in a gain – a neomorphic function to produce high amount of R2-hydroxyglutarate (R2-HG). The production of R2-HG is at a large extent responsible for the oncogenic effect of mutant IDH1–2: this oncometabolite acts as a competitive inhibitor of α -ketoglutarate-dependent dioxygenase reactions, catalyzed by many enzymes playing a key role in the biology of HSCs and cancer, such as TET2; following these effects, IDH1–2 mutants perturb the epigenetic state of cells [56]. Mutant IDH1–2 have also oncogenic effects that are independent of R2-HG: in fact, at variance of IDH1–2 which produce NADPH, mutated IDH1–2 produce NADP⁺, which drives an increase of reactive oxygen species, altering hematopoietic differentiation and promoting leukemic transformation [56].

Various experimental studies have directly supported an oncogenic role of IDH1–2 mutations in AMLs [53,54]. Particularly, mutant IDH1 is not sufficient to cause leukemia as a single oncogenic hit and additional mutagenic events are required [56]. Thus, co-expression of mutant IDH1 with HOXA9 in mouse bone marrow cells induced the rapid development of a monocytic leukemia in mice [57].

Studies in these mice have provided clear evidence that R2-HG acts, in cooperation with HoxA9, as a driver of the expansion of many preleukemic and leukemic clones; however, mutant IDH1 protein is a stronger oncogene than R2-HG, thus suggesting that the mutant IDH1 acts both through a R2-HG-dependent and -independent manner and both these

activities need to be inhibited to obtain a full antileukemic effect [58].

Therefore, all these studies have supported a key role of IDH mutants in the development of some AMLs through and alteration of the epigenetic program related to inhibition of dioxygenase enzymes that modify methyl-cytosine to hydroxymethylcytosine and histone tail methylation. According with these findings, it was hypothesized that small molecules acting as selective inhibitors of IDH-mutant enzymes could operate a therapeutic reprogramming of leukemic cells, with reversion of the abnormal epigenetic program. Thus, various IDH inhibitors have been synthesized and characterized in preclinical studies and some of them undergo a program of clinical development.

Potent chemical inhibitors of mutant IDH1–2 have shown remarkable pharmacologic effects on IDH-mutant leukemic cells, both in animal models and in *in vitro* assay on primary leukemic blasts.

Thus, the potent IDH2-mutant inhibitor AGI-6780 induced the differentiation of TF1 erythroleukemia and IDH2-mutant primary human AML cells *in vitro* and markedly inhibited R2-HG production by these cells [59]. The biologic effects of the IDH1 inhibitor GSK321 on primary IDH1-mutant AML blasts were evaluated showing: a decrease of intracellular 2-HG levels; an initial increase of viable cells, followed by a late reduction of cell viability and increase of apoptosis; a progressive abrogation of the myeloid differentiation block, associated with induction of granulocytic differentiation of leukemic cells, as shown by cell morphology and membrane antigen expression; a decrease of stem-like leukemic cells, due to induction of their differentiation; reduction of AML blasts *in vivo* in mice xenotransplanted with IDH1-mutant AML cells; overall DNA cytosine hypomethylation compared with untreated cells, with consequent upregulation of genes associated with progenitor growth and differentiation, such as CD38 and myeloperoxidase [60].

AG-120 is a IDH1-mutant-specific inhibitor, orally available, currently being evaluated in multiple clinical trials including various types of AML patients and IDH1 mutant-positive solid tumors (Table 4). Clinical results from an ongoing Phase I dose-escalation trial (NCT02074839) in relapsing/refractory AML patients were presented at the 2015 American Society of Hematology Annual Meeting: 66 patients with

Table 4. IDH inhibitors at various stages of clinical development.

Inhibitor	Structure	Specificity	Dosing (mg)	Clinical studies	Company
AG-120 (Ivosidenib)		IDH1	100–500 (b.i.d.) 500–1200 (q.d.) 500 for Phase II studies	Phase I/II	Agios Pharmaceuticals Inc.
AG-221 (Enasidenib)		IDH2	30–150 (b.i.d.) 50–450 (q.d.) 100 for Phase II studies	Phase I/II	Agios Pharmaceuticals Inc.
AG-881	N.A.	IDH1/IDH2	5–100 (q.d.)	Phase I	Agios Pharmaceuticals Inc./Celgene
IDH305	N.A.	IDH1		Phase I	Novartis

b.i.d.: Twice per day; N.A.: Not available; q.d.: Once per day.

relapsing/refractory AMLs bearing the IDH1 mutant were treated with AG-120 and well tolerated this drug up to 1200 mg once per day; among these patients a 36% of overall response rate was observed, with a complete remission rate of 18% [61]; among responders the median duration of response was 5.6 months, including responses ≥ 11 months [61]. The follow-up of this study will involve three expansion arms of patients, including relapsing/refractory AMLs, untreated AMLs and other IDH1 mutation-positive advanced hematologic malignancies [61].

AG-221 is an orally available, selective inhibitor of the IDH2-mutant enzyme (Table 4). AG221 has received the designations of orphan drug and fast track from the US FDA and is currently under investigation in various types of patients bearing IDH2-mutated cancer, including relapsing/refractory AMLs, older relapsing/refractory AML patients (comparative study with conventional standard regimens, in frontline therapy of AML patients in combination with chemotherapy or 5'-azacytidine). Preclinical studies have supported a robust antitumor activity of AG-221, documented in primary AML blasts carrying the IDH2-R140Q mutation in xenograft models, where the administration of the drug conferred a survival advantage and induced the *in vivo* differentiation of the grafted leukemic cells [62].

Recent preliminary results of the ongoing first-in-human Phase I/II dose-escalation study (NCT01915498) with AG-221 (Table 4) have been reported, showing in 128 relapsing/

refractory AMLs evaluable for clinical efficacy 41% objective responses, with a median response duration of 6.0 months; the response rates were independent of the number of previous therapeutic regimens and of the molecular type of IDH2 mutation (either R140Q or R172K). Eight treated patients went to bone marrow transplantation [62]. It is important to note that in the AML patients responding to AG-221 treatment increases in the absolute neutrophil counts occurred rapidly during the first cycle of drug administration, suggesting that despite the persistence of the mutant clone, differentiation into mature myeloid cells occurred [63].

The AG-881, a pan IDH inhibitor, and IDH 305, a IDH1 inhibitor, are also under evaluation in Phase I studies for the treatment of AML patients (Table 4).

In addition to the direct targeting of IDH-mutant enzymes, other biochemical pathways can be efficiently targeted in IDH-mutant leukemic cells. In this context, particularly interesting was the study carried out by Chan *et al.* based on a large-scale RNA interference (RNAi) screen to identify genes that are synthetic lethal to the IDH1-R132H: in this screening, they identified the BCL-2 gene [64]. In line with these observations, both IDH1- and IDH2-mutant AML cells were more sensitive to BCL-2 targeting than IDH-WT AML cells with the small-molecule inhibitor ABT-199, which induced apoptosis of leukemic cells [64]. The engraftment of IDH-mutated AML cells into immunodeficient mice

was inhibited by ABT-199 [64]. These findings indicate that the IDH1/2 mutation status identifies a subgroup of AMLs that are responsive to pharmacologic BCL-2 inhibition [64]. Very interestingly, in a Phase I clinical trial (NCT 01994837), the BCL-2 inhibitor ABT-199 induced complete response in five of 32 AML patients, most of whom had relapsed/refractory leukemic disease and 3/5 of these complete responders were IDH-mutant AMLs [65]. Particularly, three of 11 patients with IDH mutations achieved a complete response following treatment with ABT-199 [65]. Obviously, the number of treated patients is too low to support any conclusion about the clinical efficacy of BCL-2 inhibitors in IDH-mutant AMLs; however, in spite of these limitations, these preliminary observations suggest that patients with IDH mutations may be particularly sensitive to ABT-199.

It is very important to point out that in the therapeutic targeting of IDH-mutant AMLs, the mutant IDH not only represents the target of the therapy but also a suitable biomarker both for the identification of the AML subset and for the monitoring also of minimal residual disease after remission induction chemotherapy [66] or after HSCT [67].

However, a recent report raised some caution in the detection of mutant IDH as a valuable marker of MRD in IDH-mutant AMLs. In fact, Weseman *et al.* have explored the dynamic change of IDH2^{R140Q} mutant following chemotherapy for AML and have observed in some patients the reconstitution of IDH2^{R140Q}-mutated, functionally normal, clonal hematopoiesis following successful chemotherapy [68]. On the basis of these findings, it was suggested that at least in some mutant-IDH patients may represent a marker of cellular clones of indeterminate potential leading to clonal expansion, not associated with leukemic transformation [69].

Targeting of membrane antigens selectively/preferentially expressed on leukemic progenitor stem cells

A different strategy of targeting leukemic cells consists in the choice not of a molecular target directly linked to an AML subtype, but of a membrane protein preferentially or selectively expressed at the level of leukemic cells and particularly, of leukemic progenitors and stem cells. One important potential advantage of this type of drugs is related to their mechanism of action

and efficacy, which are not dependent on AML mutational complexity.

Rare leukemic stem/progenitor cells (LSPCs) initiate and maintain the leukemic process; their existence is directly supported by functional studies involving xenotransplantation of bone marrow and blood cells of AML patients into immunodeficient mice. Like normal HSCs, LSPCs possess the double capacity of self-renewal and differentiation; however, their differentiation capacities are limited, compared with those of normal HSCs [70]. LSPCs have been the object of intensive studies aiming to their characterization at cellular and molecular levels; in this context, particularly relevant was the demonstration that several membrane markers are selectively or preferentially expressed on LSPCs, compared with normal HSCs/hematopoietic progenitor cells (HPCs) (reviewed in [71]). These membrane markers represent a precious tool for the purification, identification and characterization of LSPCs, for the monitoring of the various antileukemic treatments at the level of the LSPC compartment, and for the identification of relevant therapeutic targets. Concerning this last point, the most promising therapeutic targets are CD33 and CD123.

CD33 targeting

CD33 or sialic acid-binding Ig-like lectin 3 is a transmembrane receptor expressed on myeloid cells at the level of multipotent and unipotent myeloid progenitors, and maturing compartments of the granulocytic and monocytic lineages. However, CD33 is not expressed on pluripotent HSCs. A high CD33 expression is observed in the large majority of AMLs and, importantly, the number of CD33 molecules on the membrane of leukemic cells is higher than that observed on normal bone marrow myeloid precursors [71]. The level of CD33 expression was investigated in various AML subtypes, providing evidence that its expression is particularly high in AMLs displaying NPM1 and FLT3-ITD mutations, while usually low levels are observed in AMLs bearing core-binding factor translocations. CD33 seems to be a good leukemic target because its expression is observed on leukemic cells at various stages of differentiation, including immature leukemic cells (CD34⁺/CD38⁻ or CD34⁺/CD38⁻/CD123⁺); importantly, xenotransplantation assays in immunodeficient mice have shown that isolated CD33⁺ leukemic

cell population contains, in most of AMLs, LSPCs [72].

Gemtuzumab ozogamicin (GO) is a recombinant humanized antibody anti-CD33 mAb, covalently attached to the cytotoxic antibiotic calicheamicin through a bifunctional linker. Giving the promising effects observed in relapsed AML patients treated with GO, this drug was approved by the FDA in 2000. However, during postmarketing, some clinical studies do not have supported clinical benefit for relapsed AML patients treated with GO. Based on these results, the Pfizer Company voluntarily withdrew GO from the market in mid-2010. This was, however, a rash decision in that subsequent studies have shown that the inappropriate analysis of clinical data masked the clinical benefit deriving from GO administration: in fact, analysis of the whole population of AML patients treated or not with GO in association with the standard induction chemotherapy showed no benefit deriving from GO administration; however, stratification of patients by cytogenetics clearly showed that GO administration clearly improved survival, in patients with favorable cytogenetics, but not in those with poor-risk disease [73–75]. The meta-analysis of individual patient data from five randomized controlled trials showed that GO treatment significantly reduced the risk of relapse and improved overall survival at 5 years; the absolute survival benefit was especially apparent in patients with favorable cytogenetic characteristics, but also in those with intermediate characteristics [76]. Furthermore, two recent Phase III clinical trials showed clinical benefit for older AML [77] and pediatric AML [78] patients treated with GO alone or GO+ conventional chemotherapy [77]. In both these studies, the clinical benefit correlated with the level of CD33 antigen expressed on leukemic cells [77,78].

The observation of a clear clinical benefit observed in some patients treated with GO provided support to the value of CD33 as a therapeutic target in AML and to the idea that the withdraw of this drug from the market was not appropriate. Thus, a new CD33 targeting compound was developed, an immune-conjugated anti-CD33, SGN-CD33A. This compound contains a humanized anti-CD33 mAb with engineered cysteines, conjugated to a synthetic DNA cross-linking pyrrolobenzodiazepine dimer through a protease-cleavable linker: the molecule contains about two pyrrolobenzodiazepine

dimers per antibody, is readily endocytosed by CD33⁺ cells and exerts a marked cytotoxic effect, causing DNA damage, cell-cycle arrest and apoptotic cell death; furthermore, SGN-CD33A was more potent than GO against CD33⁺ leukemic cells, including primary AML blasts and was active against AML models with multidrug-resistant phenotype [79]. Importantly, SGN-CD33A appears to have the antileukemic activity of GO without liver toxicity [80].

SGN-CD33A is under evaluation in several clinical trials. An initial Phase I study (NCT02326584) showed that SGN-CD33A administration to AML patients who have relapsed after initial remission is safe, well tolerated and exhibits the expected pharmacodynamic activity, inducing blast clearing in 47% of enrolled patients [81].

Very encouraging are the results of an ongoing Phase I study evaluating SGN-33A in combination with hypomethylating agents in older AML patients, not candidates for intensive chemotherapy or allogeneic stem cell transplantation [82,83]. These data were presented at the 2015 American Society of Hematology Annual Meeting [82] and at the 2016 European Hematology Association Meeting [83]. On 49 evaluable patients, 76% of responses were observed, 71% being complete responses; the median overall survival for the first treated patients was 12.75 months, with a median follow-up of 12.5 months; the responses were equally observed in high-risk and intermediate-risk patients [83]. Although these results are preliminary and obtained in the context of a Phase I study, are clearly favorable in comparison to that seen historically in these patients with current standard of care alone. These observations have supported the Phase III CASCADE study (NCT019002329), which aims to evaluate SGN-33A in combination with hypomethylating agents in AML patients.

Although the clinical targeting of CD33 is a promising strategy for the development of new therapeutic approaches of AML, its real impact will require the assessment through carefully randomized clinical trials in selected subpopulations of AML patients who may benefit from this treatment.

CD123 targeting on AML

CD123 (the alpha chain of IL-3R) is frequently overexpressed in AMLs and represents a marker for LSC. This topic was recently reviewed and analyzed in detail [71,84].

CD123 was clearly overexpressed in about 50% of the AMLs at the level of the bulk blast cell population [85]; CD123 is clearly expressed on CD34⁺CD38⁻ leukemic cells, while the normal CD34⁺CD38⁻ cell population scarcely expresses this membrane antigen [86]; CD123 expression on AML blasts confers a survival/growth advantage to leukemic cells and is related to a negative outcome [85,87].

Furthermore, CD123 present on CD34⁺ cells is a valuable marker of MRD, thus suggesting that the targeting of this membrane receptor could represent a therapeutic strategy for eradicating in some AML patients the residual leukemic cell population postchemotherapy [88,89].

Since CD123 is a membrane receptor, its targeting can be achieved by using its natural ligand, IL-3, or specific mAbs. Thus, concerning the use of IL-3, the basic approach until now used consisted in the conjugation of IL-3 molecule with an antileukemic drug with any type of molecule exerting a cytotoxic effect. One compound with these properties is DT₃₈₈ IL-3 fusion protein containing the catalytic and translocation domains of diphtheria toxin fused to human IL-3 [90]. A variant of this fusion toxin was obtained by fusing the diphtheria toxin with a variant IL-3 with increased binding affinity (DT₃₈₈ IL-3[K116W]) [91]. On a molar basis, DT₃₈₈ IL-3[K116W] was clearly more active than DT₃₈₈ IL-3 in mediating leukemic cell killing [91]. In spite of these differences, both DT₃₈₈ IL-3 and DT₃₈₈ IL-3[K116W] are able to interact *in vitro* and *in vivo* with cells expressing on their surface IL-3Rs and to induce a cytotoxic effect: importantly, the extent of cytotoxicity induced by these two compounds is directly correlated with the level of IL-3R expressed on the surface of target cells [92,93]. Preclinical studies have shown that DT₃₈₈ IL-3 administration is well tolerated *in vivo*, up to 100 µg/kg [94].

These promising data have represented the appropriate background for the development of IL-3 cytokine toxin fusion protein as a drug (SL-401), to be evaluated in Phase I clinical trials. SL-401 was tested in Phase I clinical studies in heavily pretreated AML patients (NCT 02270463): a report on 70 AML patients showed two complete responses and five partial responses and an improved survival compared with historical controls [95]; interestingly, in some treated AML patients, a stable disease condition was observed for more than 1 year [95]. An ongoing

expansion study is planned in AML patients, using SL-401 at 12 µg/kg per day [96].

It is important to note that Phase I/II studies have supported clinical efficacy of SL-401 in inducing the killing of blastic plasmacytoid dendritic cell neoplasms, with a high percentage of the treated patients achieving complete remissions [97].

The second strategy consisted in the development of mAbs that interact with high affinity with the human IL-3R and block the binding of IL-3 to this receptor. The mAb 7G3 recognizes the N-terminal domain of the human IL-3R alpha chain and functions as a potent and specific IL-3R antagonist [98]. This antibody exerted a potent inhibitory effect *in vitro* and *in vivo* on the growth of myeloid leukemia cells, importantly, this antibody inhibited also the fraction of LSPCs, while it minimally affected the growth of normal HSCs [98]. Starting from the 7G3 antibody, through a 'humanization' process, an increase of antibody receptor affinity and Fc region engineering to increase the affinity binding for the Fc receptor CD16, it was developed the CSL362 antibody [99]. CSL362 displays the properties of the original antibody from which it was derived, particularly for that concerns its capacity to inhibit the growth of CD123⁺ leukemic cells, including the LSPC fraction [99]. Interestingly, in view of therapeutic applications, preclinical studies have shown a remarkable synergism of CSL362 with antileukemic drugs in various leukemia models [100].

CSL362 antibody was tested in a Phase I study (NCT01632852) to obtain preliminary data about its antileukemic activity in a group of 40 refractory AML patients: since only two patients have shown a response to this treatment, it was concluded that the use of this drug alone in patients with refractory disease is an insufficient therapeutic strategy [101]. A second Phase I study (NCT 0272145) was carried out in a group of AML patients who have achieved a first or second complete remission, but are not candidate for HSCTs and are at high risk of relapse. Interestingly, 11 of the treated patients displayed MRD⁺ at baseline: following the treatment with CSL362, four of these 11 patients converted to MRD negativity; the four patients who converted to MRD⁻ maintained complete remission at week 24, while the seven MRD⁺ patients who did not convert to MRD⁻ relapsed prior to week 24 [102]. The conversion from MRD⁺ to MRD⁻ observed in four of 11 patients, with complete remission maintained at

week 24, suggests possible eradication of residual LSCs with CSL362 treatment [103].

Other approaches of CD123 targeting are based on the development and therapeutic use of bispecific mAb and T-cell expressing CD123 chimeric antigen receptors. The development of these innovative approaches, still at the experimental stage, was recently reviewed [104].

Conclusion

The overall results from large groups of AML patients have not materially changed over the past 15 years despite the incorporation of some targeted agents for several AML subtypes mainly because these studies were limited at an initial clinical stage and were performed on nonideal populations of patients (stage of disease) and often involved the therapeutic use of targeted agents in monotherapy. Consequently, targeted agents have still to enter the clinical practice for AML treatment.

The new molecularly targeted agents here briefly analyzed, all have clinical activity as single agents and may increase the overall survival in patients with AML. However, it is likely that the best responses will be seen when these agents are used in the optimal patient subtypes and in the optimal disease stage and are combined with conventional induction chemotherapy or with each other. Thus, it is reasonable to envisage future developments leading to the routine use of drugs, such as FLT3 inhibitors and IDH inhibitors or SGN-CD33A in induction, consolidation and maintenance therapy after consolidation. Preliminary results derived from ongoing clinical trials suggest that some of these molecularly targeted therapies, administered during the MRD stage could provide a fundamental tool to attempt to eradicate the residual leukemic clones, at least in some AML patients. Furthermore, the development of clinical trials of molecularly targeted agents offers the

unique opportunity of evaluating the effects on the target at molecular and cellular level and to rapidly define the mechanisms responsible for sensitivity/resistance and to design strategies to try to circumvent these drug resistances.

Future perspective

It is reasonable to predict a future with a consistent development and introduction in clinical practice of targeted therapies in AML. Recent advances in molecular classification of AMLs have led to the identification of pathogenic pathways that can be exploited with targeted agents and rational drug combinations. The future developments in this field will involve first the demonstration that some targeted agents have clear clinical activity and prolong the overall survival of selected subtypes of AML patients when administered alone or with conventional chemotherapy. A fundamental issue will be represented by the disease stage treated by targeted therapies: the actual evidences suggest potentially important applications of some selected targeted therapies as a tool to eradicate the leukemic residual disease. In this context, the identification and continuous monitoring of some suitable biomarkers (i.e., NPM1 mutations) of disease status will offer the unique opportunity to rapidly gain precious information about the possible clinical efficacy of these treatments.

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