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Chapter

Mechanisms of Resistence of New Target Drugs in Acute Myeloid Leukemia

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Abstract

New drugs targeting single mutations have been recently approved for Acute Myeloid Leukemia (AML) treatment, but allogeneic transplant still remains the only curative option in intermediate and unfavorable risk settings, because of the high incidence of relapse. Molecular analysis repertoire permits the identification of the target mutations and drives the choice of target drugs, but the etherogeneity of the disease reduces the curative potential of these agents. Primary and secondary AML resistance to new target agents is actually an intriguing issue and some of these mechanisms have already been explored and identified. Changes in mutations, release of microenvironment factors competing for the same therapeutic target or promoting the survival of blasts or of the leukemic stem cell, the upregulation of the target-downstream pathways and of proteins inhibiting the apoptosis, the inhibition of the cytochrome drug metabolism by other concomitant treatments are some of the recognized patterns of tumor escape. The knowledge of these topics might implement the model of the 'AML umbrella trial' study through the combinations or sequences of new target drugs, preemptively targeting known mechanisms of resistance, with the aim to improve the potential curative rates, expecially in elderly patients not eligible to transplant.

Keywords: acute myeloid Leukemia, FLT3 inhibitors, IDH inhibitors, BCL2 inhibitors, mechanisms of resistance, immunotherapy, target therapy

1. Introduction

The better knowledge of leukemogenesis has led in the last few years to approval of new target drugs for AML treatment. The availability of these drugs has dramatically changed the AML treatment guidelines, supported by the evidence of their efficacy on a molecular driven basis approach. Neverthless primary resistance and clonal evolution leading to adaptive resistance is a recurring theme even in this setting.

Actually acute myeloid leukemia (AML) is the result of a multi-step sequence of events resulting in impairment of lineage differentiation, hematopoiesis and enhanced self-renewal. Somatic mutations contribute to AML pathogenesis in different manner. Analysis of healthy population exomic and genomic sequencing [1] showed a correlation between pre-leukemic somatic mutations (IDH1/2, SRSF2, U2AF1, TP53, RUNX1, PPM1D) and subsequent development of AML, as first step

process towards leukemogenesis. The subsequent acquisition of mutations appeared to be related with different AML phenotypes. The Cancer genome atlas research network [2] identified eight different genetic pathways responsable of leukemogenesis in 200 adult patients, shown in **Table 1** (trancriptor factor genes fusion and hyper-expression; nucleophosmin 1 delocalization; tumor suppressor genes inhibition; mutations of: DNA-methylation related genes, activated signaling genes, chromatin-modifying genes, cohesin-complex genes, spliceosoma-complex genes). Afterwards Papaemmanuil et al. [3] identified three other molecular subgroups including: IDH2R172 mutation in 1% of AML, mutually exclusive with NPM1, associated with more severe alterations of metabolic activity in comparison to other IDH2 mutations; CCAAT/enhancer binding protein alpha (CEBPA) biallelic mutated AML and inv3 or t(3;3) AML with MECOM (EVI1) and GATA2 mutations. Furthermore Ibanez et al. [4] analyzed 100 patients with normal karyotype AML, lacking NPM1, FLT3, and CEBPA mutations, identifying thirteen seed-genes involved in leukemogenesis with a mean of 4.89 mutations per sample. The network analysis showed a high heterogeneity of gene mutations in this setting and suggested that a specific alteration could not be essential for leukemogenesis, as the interaction between several deregulated pathways.

Mechanisms of action	Class of mutations	Mutations/translocations (prevalence)
Transcription deregulation and impaired hematopoietic differentation.	Class 1 Transcription factor fusions[*]	t(8;21), t(16;16), t(15;17), MLL fusions (18%)
Aberrant localizationn of NPM1 and MPM1-interacting protein.	Class 2 NUCLEOPHOSMIN 1	NPM1 mutations (27%)
transcriptional deregulation and impaired degradation through the mouse double minute 2 homolog (MDM2) and the phosphatase and tensin homolog (PTEM).	Class 3 Tumor suppressor genes	TP53, WT1, PHF6 (16%)
Epigenetic modification and accumulation of 2-hydroxyglutarate (2HG) which deregulates DNA methylation	Class 4 DNA-methylation-related genes: DNA hydroxymethylation	TET2, IDH1, IDH2, DNA methyltransferases DNMT3A (44%)
Proliferative advantage through the RAS– RAF, JAK–STAT, and PI3K–AKT signaling pathways.	Class 5 Activated signaling genes	FLT3, KIT, RAS mutations (59%)
Deregulation of chromatin modification through methylation of histone or impairment of DOT1L (DOT1-like histone H3K79 methyltransferase).	Class 6 Chromatin-modifying genes	ASXL1, EZH2 mutations, MLL fusions, MLL partial tandem duplications (30%)
Transcription deregulation	Class 7 Myeloid transcription factor genes[*]	CEBPA, RUNX1 mutations (22%)
Chromosome segregation and transcriptional regulation.	Class 8 Cohesin-complex genes	STAG2, RAD21, SMC1, SMC2 (13%)
Deregulation of RNA processing.	Class 9 Spliceosoma-complex genes	SRSF2, U2AF3S, ZRSR2 (14%)

[*]*Class 1 and 7 mutations are both included in the category of mutations of transcription factors genes.*

Table 1.
Cathegories of AML mutations and their role in leukemogenesis.

The perspective of the comprehension of the eterogeneity of the disese inspired recent studies exploring genetic and transcriptomic single leukemic cell analysis with the following aims:

- find the correlation with mutations and subclonal architecture;

- define a hierarchies of leukemic clones, compared to normal hemapotoiesis;

- identify new markers and leukemic stem cell (LSC) specific gene repertoire.

The aknowledgement of these data will promote the finding of future targets for the eradication of the disease even in the biologically chemoresistant setting of LSC.

Uptoday the understanding of leukemogenesis mechanisms have led to the recent approval of FLT3, BCL2 and IDH inhibitors (FLT3i, BCL2i, IDHi).

We briefly resume the mechanisms of leukemogenesis addressed by these drugs. FLT3 tirosin kinase receptor mutations determines the constitutive activation and dimerization status of the receptor itself, indipendently from FLT3 ligand binding, and the downstream activation of leukemic cells proliperation and pro-survival pathways (RAS-NFKB, JAK–STAT, PI3K, BCL2) as showed in **Figure 1** [5]. BCL2 is an antiapoptotic protein of BCL2 family which compete with
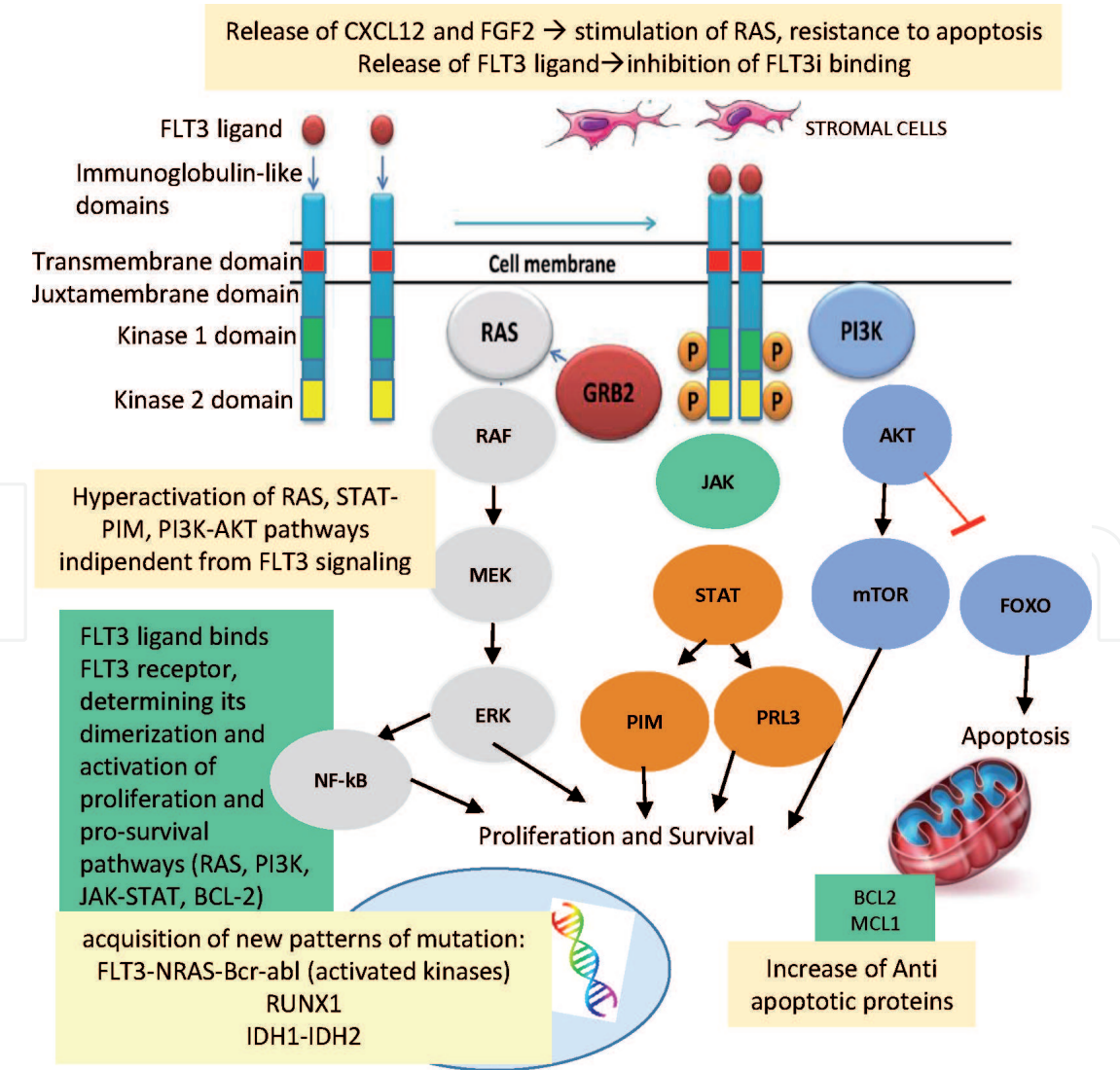

Figure 1.
FLT3 pathway (green label) and mechanisms of resistance to FLT3i (light yellow labels).

BH3 for the binding with the pro-apoptotic proteins BAK/BAX [6]. It inhibits the BH3-BAK/BAX domain and its interaction with the mitochondrial membrane, blocking the p53 dependent mitochondrial apoptosis pathway of the leukemic cell (**Figure 2**). Isocitrate dehydrogenases are cytoplasmic (IDH1) and mitochondrial (IDH2) enzymes cathalyse the reduction of a-ketoglutarate (a-KG) to citrate in krebs cycle in a NADPH-dependent way. NADPH is important for the reduction of glutathione, which in the reduced state is a major antioxidant and protects the cell against reactive-oxygen species (ROS) and other free radicals. IDH mutations have a loss of function effect, producing the accumulation of the oncometabolite R2-hydroxyglutarate (2-HG) which competitively inhibits multiple α-ketoglutarate dependent dioxygenases such as lysine (K)-specific demethylase (KDM) and ten eleven translocation methylcytosine dioxygenase 2 (TET2), causing widespread epigenetic changes with global dysregulation of gene expression and abnormal differentiation and proliferation of leukemic cells (**Figure 3**) [7]. Furthermore 2-HG activates the EglN family of prolyl 4-hydroxylases (EglN), with consequent ubiquitination and degradation of HIF1a, impairing p53 dependent apoptosis. IDH1
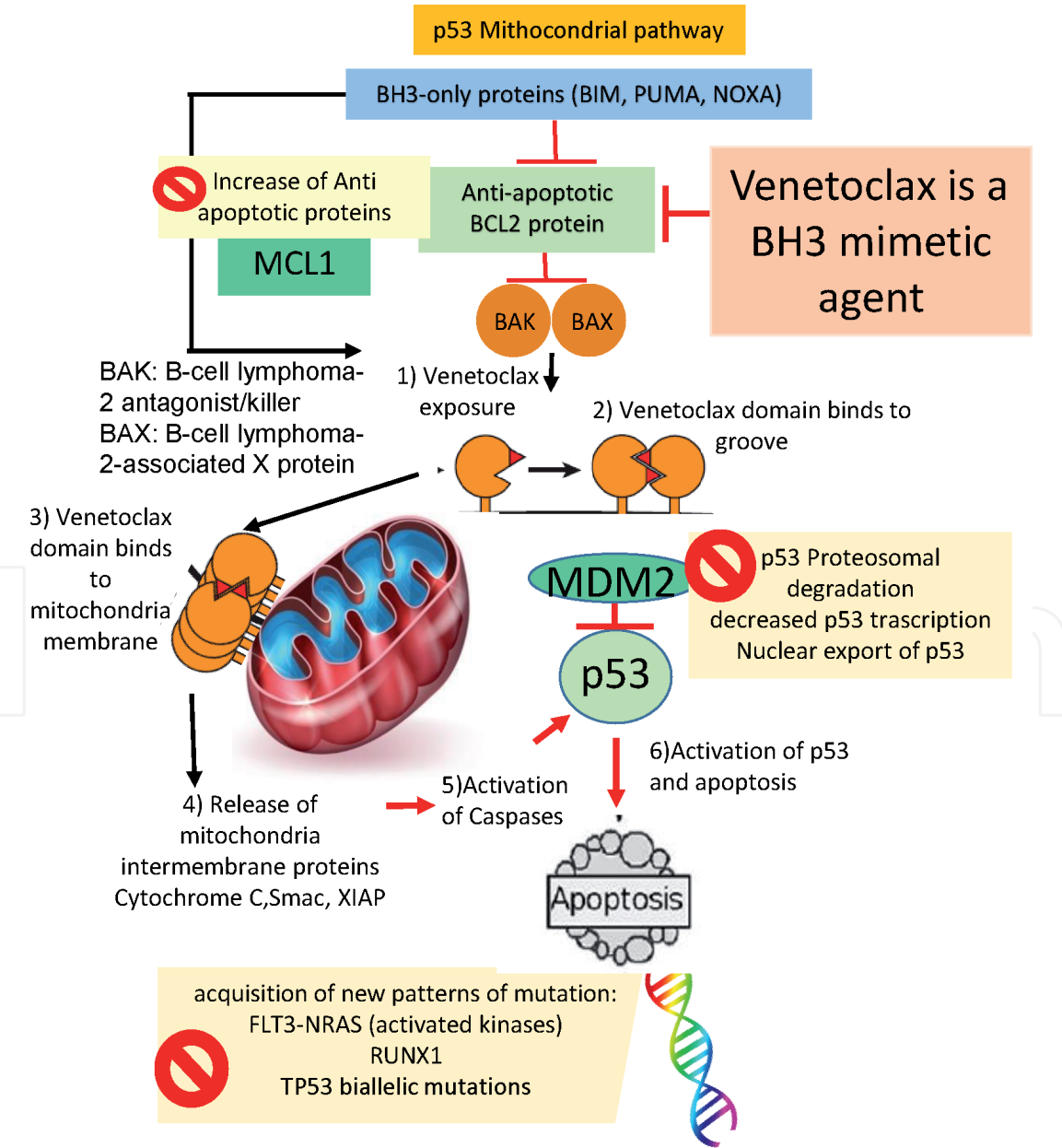

Figure 2.
p53 mitochondrial pathway and mechanisms of resistance to Venetoclax (light yellow labels).

RTK (RAS PTPN11) mutations
acquisition of secondary IDH, FLT3, RUNX1 mutations

Nucleus

Citrate

Mitochondria

Citrate

Isocitrate

IDH1

Isocitrate

IDH2

α-KG

IDH2 Mutant

IDH1 Mutant

α-KG

2-HG

2-HG

Krebs cycle

Cytoplasm

Cancer biomarker?

TET2   KDM

CH₁   CH₁

DNA

Altered epigenetic regulation

Altered differentiation and tumorigenesis

IDH mutations have a loss of function effect, producing the increasing of 2-HG which competitively inhibits multiple α-ketoglutarate dependent dioxygenases (TET2 and KDM) causing widespread epigenetic changes that result in a global dysregulation of gene expression in leukemic cell.

2-HG, 2-hydroxyglutarate; α-KG, α-ketoglutarate; IDH, isocitrate dehydrogenase; KDM, lysine (K)-specific demethylase; TET2, ten eleven translocation methylcytosine dioxygenase 2.
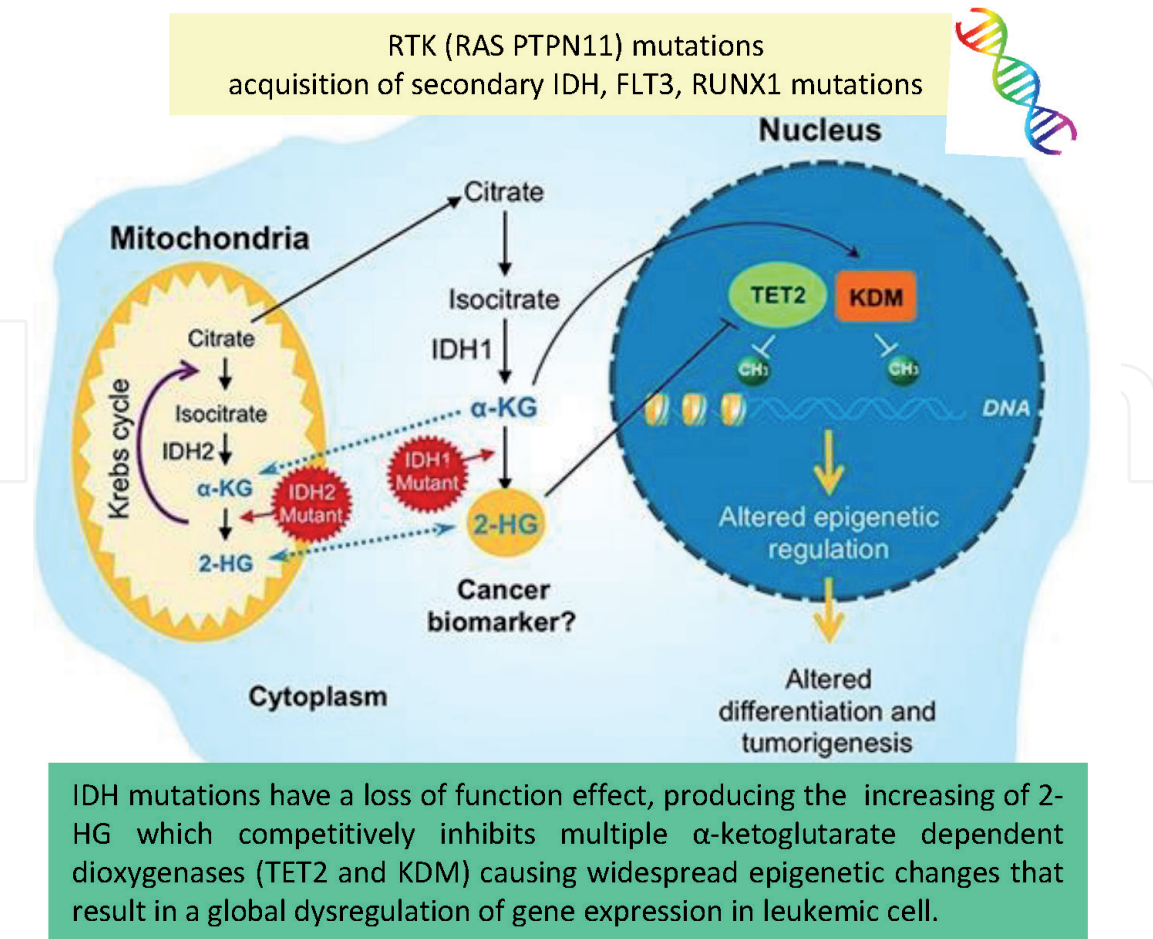
Figure 3.
Mechanism of leukemogenesis of IDH mutations (green label) and mechanisms of resistance to IDHi (light yellow label).

mutations also result in a lack of crucial metabolites including a decrease in the NADPH pool and inhibition of krebs cycle with metabolic changes conferring chemotherapy resistance of leukemic cell. At last 2-HG determines a leukemic status highly BCL-2 dependent, preventing the hypoxia mediated apoptosis, determined by cytochrome c oxidase inhibiton.

Recent studies utilizing NGS and single-cell technologies have also illustrated the complex and polyclonal nature of resistance to targeted therapeutics including FLT3, BCL2 and IDH inhibitors (FLT3i, BCL2i, IDHi) [8, 9]. Here we report the results of the principle studies aiming to analyze mechanism of primary and secondary leukemic resistance to new approved target therapies.

2. New target therapies in AML

2.1 FLT3 inhibitors (FLT3i)

FLT3 is a Tirosin Kinase receptor expressed by hematopoietic progenitors and mutated in 25-30% AML. The mutations involve two different domains: the iuxtamembrane domain (FLT3 ITD) in 20-25% AML and the tirosin kinase domain (TKD) in 5-10% AML, expecially at codon D835. They both determine the constitutive activation of the FLT3 receptor tyrosine kinase, inducing cellular proliferation and survival and inhibiting differentiation, through the activation of PI3K/AKT/mTOR pathway, with a critical role in leukemogenesis [10] (**Figure 1**).

Target drugs inhibiting FLT3 receptor showed different potency of inhibition, activity on FLT3-ITD versus TKD mutations, and on non-FLT3 targets (i.e., kinome specificity), with variable off-target toxicities [11].

Type I FLT3i (Lestaurtinib, Midostaurin, Gilteritinib, Crenolanib) are active against both FLT3-ITD and TKD mutations because they interact with the gate-keeper domain near to the activation loop or with the ATP binding site, expleting their activity on both active dimeric and inactive monomeric tirosin kinase receptor. Type II FLT3i (Quizartinib and Sorafenib) bind to the hydrophobic region adjacent to the ATP binding site only when the receptor is in an inactive form and are therefore ineffective in the forms with the FLT3TKD mutations where the receptor is always in the dimeric active form.

2.1.1 Midostaurin

Midostaurin, a type I FLT3i, also targets c-KIT, PKC, PDGFR, and VEGFR [12] and is FDA, EMA and AIFA approved for the first line treatment of FLT3 mutated (FLT3-mut) AML in association with 7 + 3 in induction and high dose Cytarabine in consolidation, on the basis of the results of the multinational, randomized phase III trial RATIFY (CALBG 10603) [13]. Midostaurin or placebo were given during induction and consolidation, and could be given for up to one year as post-consolidation maintenance, allogeneic transplant was admitted after the stop of the experimental treatment. Midostaurin was associated with a significant improvement in OS (4-year OS rate: 51.4% versus 44.3%; median OS: 74.7 months versus 25.6 months, HR = 0.78; P = 0.009) regardless of the type of *FLT3* mutation (e.g., ITD or D835 TKD) or ITD allele burden, (<0.7/≥0.7).

2.1.2 Quizartinib

Quizartinib is a type II FLT3i, but also a potent inhibitor of c-KIT, PDGFR, and RET achieving 45-50% marrow remission rates as single-agent in relapsed/refractory (R/R) *FLT3*-mut AML with an OS advantage over investigator choice salvage chemotherapy in the Quantum R-trial, a phase III randomized study of 367 patients with relapsed or refractory *FLT3*-ITD mutated AML (CRc rate 48% vs. 27%; median OS 6.2 months vs. 4.7 months, P = 0.0177) [14]. Neverthless Quizartinib failed FDA approval for this indication, due in part to concerns over treatment equipoise and robustness of OS improvement, while obtained approval in Japan in June 2019 and is being considered for approval in other countries.

2.1.3 Gilteritinib

Gilteritinib is another potent second-generation type I inhibitor with activity against AXL, a receptor tyrosine kinase that may play a role in mediating resistance to earlier generation FLT3 inhibitors [15]. Gilteritinib was found to be well tolerated as single-agent in a randomized phase III study enrolling R/R FLT3-mut AML, with marrow remission rates of 54% superior to the 22% CRc rate observed after investigator choice salvage chemotherapy (both high- and low-dose chemotherapy), with also a longer median OS (9.3 months vs. 5.6 months, HR = 0.79; P = 0.007) [16]. More patients (26% vs. 15%) were able to proceed to HSCT with gilteritinib compared with salvage chemotherapy. These results led to Gilteritinib FDA approval for the treatment of R/R FLT3-mut AML (both ITD and TKD) in November 2018.

2.1.4 Considerations on phase III trials in R/R FLT3-mut AML

Some concerns on these last two trials have been recently raised by a french retrospective analysis of 160 patients with R/R (114 relapsed and 46 refractory) FLT3-mut AML after a first-line TKI-free treatment, 92 of whom fulfilling the main criteria of the QUANTUM-R study, with CR1 durations <6 months, who received an intensive salvage regimen in 48.9% of cases achieving a 52.8% CRc rate and a bridge to transplant rate of 39.6%, superior to 27% of CR and 11% of bridge to transplant rates observed in the same setting in QUANTUM-R. The Median OS of 7 months observed in the French study was also superior to the Quantum-R OS of 4.7 months. The authors argue that the possible bias, caused by the inclusion in the control arm of patients receiving low-intensity regimens, such as low-dose cytarabine or hypomethylating agents, might compromise the results of similar phase 3 trials [17].

2.1.5 Mechanisms of resistance to FLT3i

Neverthless hematopoietic stem cell transplant (HSCT) is still necessary and recommended for the cure of the disease, since retrospective studies [18, 19] showed that HSCT improves RFS and OS and reduces incidence of relapse. The favorable predictive role of FLT3 allelic ratio in NPM1 mutated AML is still controversial due to lack of standardization of techniques and thresholds of this factor [20, 21]. Novel FLT3i might increase outcomes in this setting, but researchers have already identified multiple mechanisms of resistance as hereby reported [11].

- The acquisition of secondary mutations of single amino acids of the activation loop of the FLT3 receptor (D835, I836, D839, Y842) or of the gate-keeper residue (F691) called 'TKD' mutations are reported in 22% of FLT3 AML [22] and are responsible for the resistance specially to type II FLT3i (Quizartinib and Sorafenib) ineffective in targeting TKD mutations [23, 24].

- The activity of fibroblast growth factor 2 (FGF2) and CXCL12/CXCR4 pathways in FLT3 mutated leukemic cells can induce their chemoresistance. The increase in FGF2 is an autocrine response mechanism of stromal cells to all phenomena of hematopoietic stress, including that induced by Quizartinib. Paracrine production of FL (FLT3 ligand) by stromal cells also inhibits the action of FLT3 inhibitors with competitive mechanism, but removal of FL from stromal and leukemic cell cultures does not stop the chemoresistance process due to activation of RAS-MAPK mediated by the FGF2-FGFR1 interaction. The increase in FGF2 secreted by stromal cells has been reported to precede the relapse of mutated FLT3ITD mut AML treated with Quizartinib, through activation of the RAS–MEK/MAPK signal [25]. The combination of FGFR and FLT3 inhibitors is being studied, the rationale is represented by the inhibition of the autocrine and paracrine stimulus favoring the survival of the stromal and leukemic cells, respectively.

- Furthermore FLT3ITD mutated leukemic cells express CXCR4 and are CXCL12 dependent for growth and survival, which makes them resistant to the action of chemotherapy [26]. Activation of Nutlin-3a reduces mRNA levels and CXCL12 secretion through activation of p53 and consequent down-regulation of HIF-1 alpha. Nutlin-3a also binds MDM2 in the p53 binding domain, inhibiting its interaction with p53 which, remaining free, recovers its function.

MDM2 inhibitors such as Nutlin-3a are under study (NCT00623870) and there is a rationale for their association with FLT3 inhibitors [27].

- The activation of RAS/RAF/MEK/ERK is independent of the activation of FLT3 as it is constantly present during therapy with FLT3 inhibitors and can represent a mechanism of resistance to inhibitors in some subclones. Inhibitors of these signals could play a role in counteracting resistance to FLT3 inhibitors [28].

- The PI3K/AKT/mTOR pathway is activated and upregulated in FLT3 mut AML resistant to FLT3i, suggesting the efficacy of AKT and mTOR inhibitors in this setting. In vitro studies have shown that Sorafenib is able to inhibit FLT3 in leukemia cells of resistant patients without blocking colony formation and survival, due to a replication mechanism independent of FLT3. The GEP analysis of these cells and cell lines resistant to FLT3 inhibitors has shown downstream activation of PI3K/mTOR and in vitro and in vivo studies have shown that Gedatolisib is able, by inhibiting this pathway, to block the formation of colonies and to improve the survival of mice transplanted with Sorafenib-resistant cells [29].

- The activation of the FLT3 receptor also promotes leukemogenesis through the trigger of STAT5 and Pim-1 (serine–threonine oncogenic kinase). The FLT3ITD receptor is partially reteined in the endoplasmic reticulum as a 130 kDa protein that interacts with calnexin and heat shock protein 90 (HSP90), resulting in the upregulation of STAT5 and the consequent Pim-1 increase. Pim-1 promotes the phosphorylation at the level of serine and tyrosine 591 of the 130 kDa isoform of FLT3ITD, blocking its glycosylation and degradation, with consequent hyperactivation of calnexin, HSP90 and STAT5 by establishing a FLT3-STAT5-Pim-1 hyperactivation loop that determines the proliferation of mutated FLT3ITD cells and their resistance to FLT3i. STAT5 and Pim-1 inhibitors might therefore have a rationale in resistant FLT3 mut AML [30].

- CDK4 and CDK6 regulate the transcription of FLT3 and Pim-1 therefore CDK4 and CDK6 inhibitors, such as Palbociclib, are also useful in this setting [31].

- The upregulation of anti-apoptotic proteins Bcl-2, Bcl-xl, Mcl-1 proteins has been described in AML resistant to second generation FLT3 inhibitors. In particular, the FLT3-ITD627E mutation, located in the beta-2 sheet of the first kinase domain, induces a dramatic increase in anti-apoptotic protein MCL-1 suggesting a possible role of MCL-1 inhibitor drugs in blocking resistance to FLT3 inhibitors [32].

- The concentration in the marrow at suboptimal doses represents an additional mechanism of resistance, specially of inhibitors that have interactions with drugs metabolized by cytochrome P450 [33].

- Recently NGS and single cell analysis were performed in 41 patients with FLT3mut AML relapsing after Gilteritinib monotherapy, permitting the identification of mechanisms of resistance in 22 cases. RAS and FLT3 F691L mutations were found in 15 and 5 patients respectively and acquisition of Bcr Abl fusion protein was found in 2 samples. The FLT3 F691L mutation was more frequent in patients receiving Gilteritinib at dose lower than 200 mg, suggesting a dose dependent resistance and the importance of using higher doses to prevent this mechanism of failure [8]. FLT3 pathway and mechanisms of resistance to FLT3i are illustrated in **Figure 1**.

2.1.6 Novel FLT3i and future perspectives

Crenolanib is a potent novel type I pan-FLT3 inhibitor, effective against both ITD and TKD, but the response in monotherapy is unfortunately transient. Zhang et al. performed WES of samples from R/R FLT3 pos AML patients before and after Crenolanib, administered in a phase II study (NCT 01522469, NCT 01657682). They interestingly observed that patients previously treated with FLT3i expressed RAS mutations at baseline more frequently than naive patients and were less likely to respond to Crenolanib. They identified mutations of NRAS and IDH2 arising in FLT3-independent subclones and of TET2 and IDH1 in FLT3-subclones as possible

Combination regimen	Mechanism of action of combination agent	Mechanism rationale for combination	Clinicaltrials.gov identifier
LGH447 + midostaurin	Pim kinasi inhibitor	Pim kinasi activity mediates FLT3 inhibitor resistance; combination increases apoptosis	NCT02078609
Milademetan (DS-3032b) + quizartinib	MDM2 inhibitor	MDM2 inhibitor restore p53 tumor suppression function	NCT03552029
Omacetaxine mepesuccinate + sorafenib Omacetaxine mepesuccinate + quizartinib	Protein synthesis inhibitor	Synergistic with FLT3 inhibitors to suppress leukemic proliferation	NCT03170895 NCT03135054
Palbociclib + sorafenib	CDK4/6 inhibitor	CDK4/6 regulate transcription of FLT3 and Pim kinases (mechanism of FLT3 inhibitor resistance)	NCT03132454
SEL24 (dual pan Pim/FLT3 inhibitor)	Pim kinase inhibitor	Pim kinase activity mediates FLT3 inhibitor resistance; combination increases apoptosis	NCT03008187
Venetoclax + gilteritinb	Bcl-2 inhibitor	Upregolation of anti-apoptotic proteins (e.g. Bcl-2, BCL-xL and Mcl-1) mediates FLT3 inhibitor resistance	NCT03625505
Azacitidine + venetoclax + gilteritinib	Hypomethylating agent Bcl-2 inhibitor	Hypomethylation of target genes	NCT04140487
Vorinostat + bortezomib + sorafenib	Hystone deacetylase inhibitor (vorinostat) Proteasome inhibitor (bortezomib)	Histone deacetylase inhibitors synergistically induce apoptosis with FLT3 inhibitors; Proteasome inhibitors induce FLT3 ITD degradation through autophagy	NCT101534260

Table 2.
Trials with combinations of FLT3i and target drugs.

mechanisms of resistance. Post-crenolanib expansion of mutations associated with epigenetic regulators, transcription factors, and cohesion factors was also detected suggesting diverse genetic/epigenetic mechanisms of crenolanib resistance. Drug combinations in experimental models restore crenolanib sensitivity [34].

FLT3 F691L mutation was shown to be resistant to the majority of FLT3 TKIs including crenolanib, but not ponatinib and pexidartinib (PLX3397) [35]. In addition, a novel FLT3 extracellular mutation at K429E was detected in one patient with high VAF, which showed increased crenolanib IC50. The structural basis for the drug resistance of FLT3 K429E requires further investigations.

Given the expanding spectrum of FLT3 inhibitors FDA-approved, randomized phase III studies of conventional chemotherapy in combination with midostaurin versus gilteritinib (NCT03836209) and with midostaurin versus crenolanib (NCT03258931) are ongoing to establish wich FLT3i should be used in first line. Phase III study of gilteritinib versus placebo and phase II Crenolanib trial as maintenance after HSCT in FLT3-mutated AML are ongoing and may help to more definitively address the benefit of FLT3 inhibition in this setting (BMT CTN 1506; ClinicalTrials.gov identifier: NCT02997202, NCT02400255). Last but not least, the recent findings of intra- and extracellular mechanisms of FLT3i resistance, provided the background of ongoing trials, rationally including combinations with agents targeting specific resistance pathways. Current approaches include adding FLT3i to antiapoptotic drugs such as Venetoclax or milademetan or with drugs inhibiting other target such as PIM kinasi or CDK4/6. The addition of chemotherapy or hypomethylating agents (HMA) to this backbone could be the following step in patients eligible and not to intensive chemotherapy. **Table 2** reports the ongoing studies exploring association of FLT3i with other drugs.

2.2 Venetoclax

One of the possible mechanisms of leukemogenesis is represented by the functional loss of p53 or by an altered balance of antiapoptotic and proapoptotic protein expression [36]. Apoptosis is controlled by two parallel pathways, intrinsic and extrinsic, leading to activation of intracellular caspases, ending with cell death. The intrinsic pathway is under the control of the BCL2 family proteins, including antiapoptotic proteins (e.g., BCL2, BCL-XL, and MCL1), proapoptotic BH3-only proteins (e.g., BIM, BAD, PUMA, and NOXA), and proapoptotic effector proteins (e.g., BAK and BAX) (**Figure 2**). Leukemic cells showed to overexpress BCL2 making of Venetoclax, an oral inhibitor of BCL2, an ideal target therapy. Venetoclax acts as BH3 mimetic protein and restors apoptosis without interacting with other antiapoptotic proteins such as BCL-XL or MCL-1. Based on phase II study outstanding results [37] venetoclax has been approved by FDA, EMA and AIFA for first line treatment of elderly AML over 75 years or unfit for intensive chemotherapy. The trial associated venetoclax at the doses of 400 or 800 mg daily in combination with either decitabine (20 mg/m^2, days 1-5, intravenously [IV]) or azacitidine (75 mg/m^2, days 1-7, IV or subcutaneously) in 145 AML patients, not eligible to intensive chemotherapy, in first line. CRc (CR and CRi) rate was 73% with a median duration of 11.3 months and a median overall survival of 17.5 months.

2.2.1 Mechanism of resistance to Venetoclax

Avoidance of apoptosis and the acquisition of BCL-2 mutations such as BCL2 Gly101Val are among the mechanisms currently identified for resistance in chronic lymphocitic leukemia. MCL-1 inhibitors appear to bypass this mutation in preclinical studies and preliminary clinical studies with these agents are ongoing [38].

Mechanisms of resistance to Venetoclax, have been further investigated in AML. A recent study of DiNardo [39] performed NGS at baseline and relapse and follow-up and single cell analysis at baseline and relapse in 81 AML patients receiving HMA (N: 58) or low dose cytarabine (LDAC) (N: 23) with Venetoclax in frontline (NCT02287233 and NCT02203773) at the MD Anderson Cancer Center (Houston) or at the Alfred Hospital (Melbourne). The median age of this elderly cohort was 74 years (range, 62-87 years). The HMA group excluded prior HMA therapy. In contrast, the LDAC group included patients with prior HMA exposure. The target dose of venetoclax was also different in the 2 studies (HMA study: 400 mg/day; LDAC study: 600 mg/day).

Twenty-five cases had adaptive resistance, representing 31% of the total cohort of 81 patients. The median time to relapse was 6.4 months (95% confidence interval, 4.5-10.6 months); 5 patients relapsed after 12 months. To identify dynamic molecular changes indicative of adaptive resistance, the VAFs of individual mutations were compared at diagnosis, in remission, and at relapse to identify clones expanded at relapse. Two important findings emerged: progressive expansion of clones with activated kinases, particularly FLT3-ITD, and in other cases, selection of clones with likely biallelic perturbation of TP53. The single cell analysis of the relapsed clones also showed the selective impact of the expansion of FLT3-ITD or other kinase (CBL, NRAS) in mediating resistance with FLT3-ITD loss of heterozygosity (LOH) at relapse.

In contrast, NPM1mut and IDH2mut were associated with high rates of response and durable remissions. In NPM1mut AML, measurable residual disease (MRD) was eliminated in most cases. Median OS for patients with either NPM1mut or IDH2mut was not reached, with 2-year OS of 71.8% and 79.5%, respectively. In the durable remission group, DNMT3A mut was present in 44% of cases (8/18), and 6 out of 8 of these cases were among patients with concurrent NPM1 or IDH2mut. The association between IDH1mut and prognosis was less clear. There were 2 IDH1mut cases in the durable remission subgroup, and both had a co-occurring NPM1mut. Among the 7 IDH1mut cases occurring in patients with relapsing or primary refractory disease, 5 cases had a concurrent TP53, FLT3-ITD, or RAS mutation. The median OS for patients with IDH1mut was not significantly different from patients with IDH1 wild-type (WT) AML (18.3 vs. 12.7 months; P = 0.79).

Primary refractory AML had 3 patterns of resistance: TP53 abnormality, RUNX1 and activating kinase mutations (FLT3-ITD, N/KRAS, CBL, or KIT). The VAF of TP53 mutation was higher in refractory patients, while RUNX1 mutations were also found in responder patients, in association with IDH1 and SRSF2 mutations, suggesting that larger studies are needed to refine their role in resistance to Venetoclax. **Figure 2** shows the mithocondrial pathway of p53 and the mechanisms of resistance to venetoclax.

2.2.2 Future perspectives

On the basis of the results of the analysis performed by DiNardo et al. [39], a baseline molecular characterization may allow patients to be risk stratified into a favorable risk NPM1mut subgroup, where molecular MRD monitoring, and even consideration of treatment cessation, could be employed within a future clinical trial. Patients with IDHmut could be considered for postremission IDH inhibitor maintenance-based approaches, aimed at eradicating residual molecular disease, and patients with FLT3-ITD mutations could also benefit from the addition of targeted FLT3 inhibitors to prevent failures. Relapsed and refractory patients with TP53 mutations actually still represent an unmet medical need. Clinical trials incorporating new agents targeting TP53mut/del(17p) drug resistant clones should be pursued. APR 246 showed encouraging results in a phase I/IIa clinical trial of patients with hematological malignancies or prostate cancer [40] due to

the reactivation of the disrupted TP53 through the conversion to methylene qui-nuclidinone (MQ), a Michael acceptor that reacts with cysteines in the p53 core domain restoring its activity [41]. However, the mechanism by which APR-246/MQ reactivates mutant p53 is not fully understood. In early results from an ongoing phase Ib/II study in patients with high-risk *TP53*-mutated MDS or oligoblastic AML (20–30% blasts), the combination of APR-246 and azacitidine resulted in a composite CR, CRi, and morphologic leukemia-free state (MLFS) rate of 100% (11 of 11 evaluable patients), with 82% CR and 72% of responders having undetectable *TP53* mutation by nextgeneration sequencing [42].

Transcriptomic analysis after run-in of single-agent APR-246 confirmed on-target effects, including transcriptional activation of p53 targets. A phase III randomized study of azacitidine with or without APR-246 in MDS and AML with 20–30% blasts is ongoing (NCT03745716).

Preclinical studies showed sinergy between MCL1 inhibition and venetoclax [43] and therefore MCL1 inhibitors are now being explored in early clinical trials, both as single agents and in combination with venetoclax. Interestingly BH3 profiling might predict sensitivity to specific BH3 agents helping the choice between BCL2 or MCL1 inhibition or a combination of both [44].

In vitro studies also showed a synergistic effect of MDM2 inhibitors in combination with BCL2i due to the downregulation of MCL1 through the inhibition of RAS–RAF–MEK–ERK pathway. In an ongoing international phase Ib study of venetoclax and idasanutlin, in patients aged >60 years with relapsed or refractory AML, the marrow remission rate was 37% (11 of 30 evaluable patients) in the entire population, and 50% (9 of 18 evaluable patients) at the recommended phase II dose of venetoclax. As with other venetoclax-based regimens, higher ratios of BCL2/BCL-XL and BCL2/MCL1 were predictive of response [45].

2.3 IDHi

IDH1 and 2 are targetable mutations occurring in approximately 20% of Acute Myeloid Leukemia (AML) patients [IDH1 (8%) and IDH2 (12%)] and are more common in the elderly (25-28%). They are usually associated with intermediate-risk cytogenetics, FLT3 and NPM1 mutations [46, 47] and mutually exclusive with the TET2 mutation [48].

Hotspot IDH1 mutations, affecting the catalytic domains, commonly involve a cysteine (R132C) or histidine (R132H) substitution for arginine. In IDH2 mutations, arginine is most often replaced by glutamine at residue 140 (R140Q) or by lysine at residue 172 (R172K) [49]. Further, IDH2 R172 and NPM1 mutations were not detected in the same patient samples [50]. The incidences of IDH1 and IDH2 mutations are equivalent and mutually exclusive [51]; however, the incidence rate of IDH2 R140Q was found to be higher than that of IDH2 R172K (9.2% vs. 2.9%) [52]. Somatic mutations in catalytically active arginine residues decrease their enzymatic activity as well as confer a gain of function activity leading to the production of the oncometabolite 2 hydroxyglutate (2-HG) instead of alpha-ketoglutarate (α-KG). 2-HG competitively inhibits the function of αKG-dependent oxygenases involved in DNA or histone demethylation, increases the production of ROS (reactive oxygen species) through the oxidation of Glutathione and determines metabolic changes interfering with NF-Kb and BCL-2 proteins, such as ten-eleven translocation (TET2) DNA methylases, and Jumonji C (JmjC) domain containing histone demethylases, resulting in global DNA hypermethylation of regulatory genes and arrested myeloid differentiation [53] (**Figure 3**). Mutant IDH has therefore become a viable target in AML treatment.

The prognostic value of the different mutation isoforms remain controversial [54]. Some AML patients with IDH mutation, especially IDH2 R172 mutation, have

a poor response to traditional chemotherapy and have a higher relapse rate [55]. Therefore, individualized treatment, specially targeted therapy for IDH mutations, may be an important option for such patients. In recent years, IDH inhibitors have shown good clinical response in AML patients. Based on phase 1/2 clinical trials, enasidenib and ivosidenib have been approved by the FDA on 1 August 2017 and 20 July 2018 for the treatment of adult R/R AML with IDH2 and IDH1 mutations, respectively [56, 57]. Ivosidenib 500 mg/day in combination with subcutaneous azacitidine was associated with an ORR of 78% (18/23) and 30% of CRs with a median OS of 12.6 months, while in patients with IDH2 mutation, enasidenib (100 mg once daily) plus azacitidine was associated with an ORR of 67% with 20.6% of CRs and median OS of 9.3 months. No patient in the ivosidenib group and one patient in the enasidenib group had progressive disease.

2.3.1 Mechanisms of resistance to IDHi

To fully characterize the mechanisms of response and relapse to ivosidenib monotherapy, Choe et al. [58] conducted a comprehensive genomic analysis of samples from a cohort of 179 patients with m*IDH1* (IDH1 mutated) R/R AML, treated in a phase 1 study with ivosidenib at the starting approved dose of 500 mg once daily (QD), confirming that RTK pathway mutations (NRAS, PTPN11) are associated with primary and secondary resistance to ivosidenib. Emergence or outgrowth of AML-related mutations, such as RTK pathway genes, and *IDH*-related mutations (comprising second-site mutations in *IDH1* and mutations in *IDH2*), which were associated with increased 2-HG, contribute to relapse after ivosidenib therapy. These various mechanisms of resistance occurred in isolation or in combination, underscoring the complex biology of treatment resistance.

Single-cell DNA sequencing analyses also found co-occurring mutations at single-cell resolution, including genes of the RTK pathway (eg, *NRAS*, *KRAS*, *PTPN11*, *FLT3*), transcription factors (*RUNX1*), chromatin/epigenetic regulators (*DNMT3A*, *ASXL1*), and splicing factors (*U2AF*, *SF3B1*). These co-occurring mutations indicate functional interplay between these genes and m*IDH1*, and reflect a more complex role of m*IDH1* during leukemogenesis or maintenance of m*IDH1*AML, such as cooperation with the constitutively activated RTK pathway to promote cell proliferation, and/or cooperation with chromatin/epigenetic regulators and transcription regulators to block cell differentiation.

The analysis of baseline mutational profiles of 101 mIDH1 AML patients [59] showed similar results with the following comutations: DNMT3A (35%), NPM1 (26%), SRSF2 (24%), ASXL1 (18%), RUNX1 (18%), NRAS (14%), and TP53 (13%); FLT3TKD (9%), FLT3ITD (2%), TET2 (14%). The achievement of CR was analogously related to lower genomic complexity with lower number of comutations in responders compared to non responders (2.8 vs. 3.7), with P < .001. RTK pathway mutations, along with an increased number of mutations, are conversely associated with primary treatment resistance.

Actually, although 2-HG–restoring mutations are a major pathway of resistance, other 2-HG-independent pathways, such as RAS and FLT3 mutations, are important and may be dominant over 2-HG restoration.

This finding is consistent with a similar work showing an association between NRAS mutations and a lower likelihood of response to enasidenib in patients with mIDH2 R/R AML [60].

Amatangelo et al. showed emergence of AML-related mutations, such as *RUNX1*, FLT3, and IDH-related mutations in patients relapsing after enasidenib in mIDH2 R/R AML. Outgrowth of mIDH1 in patients who initially had mIDH2 (isoform switching) [61, 62] and the emergence of second-site IDH2 mutations [63] were

confirmed in other series. However, these reports were based on a limited number of patients, and the frequency and breadth of resistance mechanisms have not been comprehensively characterized.

Some reports have shown that FLT3 inhibitors induce granulocytic differentiation and differentiation syndrome symptoms in some patients with *FLT3*-mutated AML [64, 65]. Thus, the combination of mIDH inhibitors with RTK pathway inhibitors, including FLT3 inhibitors, may present a rational treatment strategy. Choe et al. [58] also showed that *JAK2* mutations were associated with a high CR/CRh rate (64%), with the caveat of the limited number of patients (n = 11). Although *JAK2* mutations are often classed together with other mutations affecting MAPK pathway signaling, their different pattern of response to ivosidenib treatment depends on the distinct biology of *JAK2* mutations, such as STAT pathway activation, frequent ancestral status during clonal evolution, and association with prior myeloproliferative neoplasm. The number of patients in Choe analysis is insufficient to determine whether de novo or secondary disease has a prognostic role in the context of *JAK2* mutation. Data on additional patients with *JAK2* mutations are needed to gain a more robust picture of this patient subset. **Figure 3** summarizes the IDH pathway and the main mechanisms of resistance of IDHi.

2.3.2 Future perspectives

These findings highlight the interplay among baseline mutation profiles, response, and clonal evolution during ivosidenib therapy. The complex and polyclonal mechanism of resistance to ivosidenib has implications for mIDH1/2 inhibitor treatment strategies, and supports the use of combination therapies or sequential treatment modifications at early relapse before overt clinical progression, rather than monotherapy with mIDH1/2 inhibitors. It will also be important to understand whether or not these patterns of resistance are replicated with combination therapies. Because individual patients often show multiple resistance mechanisms at relapse, combination of ivosidenib with nontargeted agents, such as intensive chemotherapy/cytotoxic therapies, hypomethylating agents, and venetoclax (BCL-2 inhibitor), may improve responses and decrease the likelihood of relapse.

2-HG accumulation lead to cytochrome c oxidase activity, mimicking an oxygen-deprived state and decreasing the mitochondrial threshold for induction of apoptosis.

The association of IDHi to BCL2i might represents the exciting possibility of a chemotherapy free oral combination for IDH mut AML and a phase Ib/II clinical trial (NCT03471260) confirmed a surprisingly high 75% CRc rate in a cohort of 12 patients [66, 67].

2.4 Fighting policlonal resistance

As we have seen that RAS mutation are often involved in resistance to all new approved target therapies, RAS pathway–targeting agents may be useful, either concomitantly with FLT3i or IDHi to avoid primary resistance in patients with RAS mutations at baseline, or sequentially in patients who have a newly detectable RAS mutation while on therapy with a FLT3, IDH, or BCL2 inhibitor. MEK1/MEK2 inhibitors selumetinib and trametinib unfortunately did not confirm this potential efficacy since modest response rates of 17–20% were seen in relapsed or refractory RAS-mutated AML [68]. An established mechanism of resistance to MEK inhibition is the compensatory activation of PI3K–AKT–mTOR pathway.

The targeted downmodulation of pERK and pS6 was shown in a study of 23 RAS-mutated AML patients treated with trametinib plus an AKT inhibitor (GSK2141795), without producing any clinical response [69].

At last but not least immune evasion represents one of the main mechanism of resistance common to all target drugs. Actually the exhaustion of the host's own immune system contribute to cancer growth. Murine and human studies have shown association between AML and increased infiltration of T-regulatory cell and expression of immune checkpoint proteins on CD8 positive T cells, including PD-1, TIM3, and LAG3 [70] which might induce immune exhaustion and early relapse [71]. The immunotherapies with antibody targeting leukemic antigens, such as CD123 might be an effective strategy to target measurable residual disease (MRD) in maintenance therapy in high-risk AML. The IL3 receptor alpha chain, CD123, is notably expressed on leukemic stem cells (LSC) and is expressed at lower levels on normal hematopoietic stem cells (HSC) than CD33 [72].

Antibody-drug-conjugate (ADCs) and bispecific antibodies targeting CD123 have shown promising clinical activity in phase I studies and are rapidly moving to multicenter studies as single-agent expansions and in combination approaches [73, 74]. Pre therapy low levels of bone marrow CD3+ or CD8+ and overexpression of CTLA4 predict resistance to check point inhibitors [75]. These may be potential biomarkers to prospectively select patients most likely to respond. Inhibitors of "macrophage checkpoints" could be another interesting chance for immunomodulation. In particular CD47, highly expressed on LSCs, is associated with unfavorable outcomes [76]. Upregulation of CD47 on AML cells allows the binding to the signal-regulatory protein-α (SIRPα) receptor on macrophages, providing a "don't eat me" signal [77].

Hu5F9-G4 is an anti-CD47 antibody that inhibits the binding of LSC with SIRPα, promoting macrophage-mediated phagocytosis of leukemic cells [78].

The combination of Hu5F9-G4 with azacitidine in unfit newly diagnosed AML produced 64% of CR/CRi/MLFS (9/14) [79]. The study is ongoing at multiple centers (NCT03248479). Unfortunately the lack of an AML specific antigen restricted to the leukemic cell is the main reason of unsatisfactory results of vaccines and chimeric antigen receptor (CAR) T-cell therapies in this setting [80]. CD33, CD123, CLEC12A are expressed on normal cells leading to potential "on-target, off-tumor" toxicity.

3. Conclusions

In conclusion the knowledge of the mechanism of resistance might help the design of future studies with sequences (**Figure 4**) or combinations (**Figure 5**) of new target drugs. Furthermore the policlonal nature of leukemia resistance might
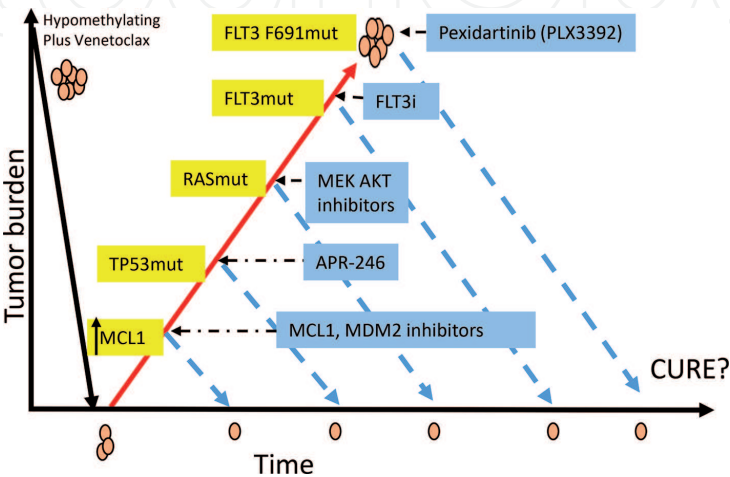

Figure 4.
Model of kinetics of chemoresistant leukemic clones after treatment with hypomethylating agents plus Venetoclax when target drugs are administered sequentially at the onset of chemoresistant clones.
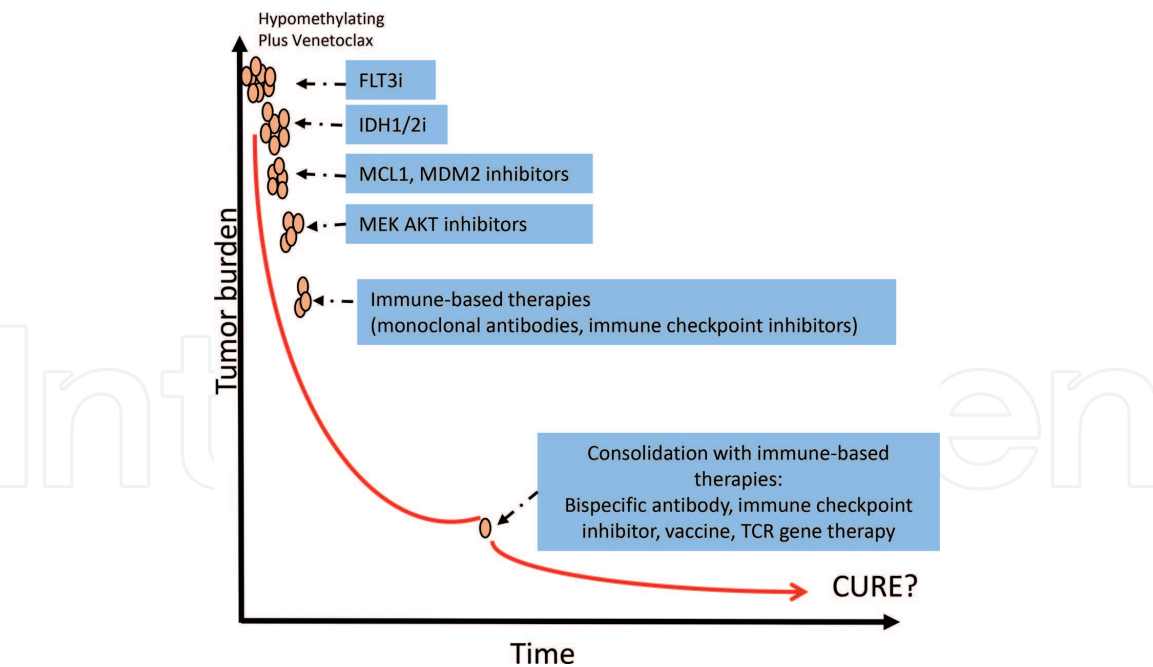
Figure 5.
Model of kinetics of chemoresistant leukemic clones after a total therapy approach including treatment with hypomethylating agents plus Venetoclax, plus combination of target drugs and immune therapies, preventively administerd to avoid emergence of chemoresistant leukemic clones. Consolidation with immune based therapies might be administered in order to reduce MRD and possibly cure AML.

reduce the efficacy of target therapies leaving a role to immune therapies such as checkpoint inhibitors, vaccines, and adoptive T-cell therapies, in decreasing the burden of residual disease. Several studies of consolidative or maintenance immune modulation in this context are ongoing [81].

These approaches may be particularly appealing in patients not eligible to allogeneic transplant. The polyclonal mechanisms of resistence to new drugs, hereby illustrated, underline an urgent need for future trials in this setting, based on total therapy approach, including initial chemotherapy or HMA with targeted or apoptosis-inducing drugs, sequentially adjusted, on the basis of emerging early clones, with immune or target-based therapies, to eradicate reservoirs of residual disease (**Figure** 5).

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