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Dual Inhibition of FLT3 and AXL by Gilteritinib Overcomes Hematopoietic Niche-Driven Resistance Mechanisms in FLT3 -ITD Acute Myeloid Leukemia

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1 **Dual inhibition of FLT3 and AXL by gilteritinib overcomes hematopoietic niche-driven resistance**
2 **mechanisms in *FLT3*-ITD acute myeloid leukemia**

3
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44

45 **Abstract**

46

47 AXL has been shown to play a pivotal role in the selective response of *FLT3*-ITD AML cells to FLT3 tyrosine
48 kinase inhibitors (TKI), particularly within the bone marrow microenvironment. Herein, we compared the
49 effect of dual FLT3/AXL TKI Gilteritinib with Quizartinib through *in vitro* models mimicking hematopoietic
50 niche conditions, *ex vivo* in primary AML blasts and *in vivo* with dosing regimens allowing plasma
51 concentration close to those used in clinical trials. We observed that Gilteritinib maintained a stronger pro-
52 apoptotic effect in hypoxia and coculture with bone marrow stromal cells compared with Quizartinib,
53 linked to a dose-dependent inhibition of AXL phosphorylation. *In vivo*, use of the MV4-11 cell line with
54 hematopoietic engraftment demonstrated that Gilteritinib was more effective than Quizartinib at targeting
55 leukemic cells in bone marrow. Finally, *FLT3*-ITD AML patient-derived xenografts revealed that this effect
56 was particularly reproducible in *FLT3*-ITD AML with high allelic ratio in primary and secondary xenograft.
57 Moreover, Gilteritinib and Quizartinib displayed close toxicity profile on normal murine hematopoiesis,
58 particularly at steady state. Overall, these findings suggest that Gilteritinib as a single agent, compared with
59 Quizartinib, is more likely to reach leukemic cells in their protective microenvironment, particularly AML
60 clones highly dependent on *FLT3*-ITD signaling.

61

62 Introduction

63

64 Acute myeloid leukemia (AML) is a myeloid malignancy carrying a heterogeneous molecular panel of
65 mutations. It participates in a blockade of differentiation and an increased proliferation of myeloid
66 hematopoietic progenitor cells. The Fms-like tyrosine kinase 3 (FLT3) is a class III receptor tyrosine kinase
67 expressed in progenitor cells. Its ligand binds and activates FLT3, inducing a strong activation of PI3K/AKT
68 and MAPK pathways (1). Twenty-five percent of AML patients display internal tandem duplication (ITD) in
69 the juxta-membrane domain of FLT3, leading to constitutive activation of its downstream signaling to which
70 is added the activation of the transcription factor STAT5 (2). This mutation, which carries an adverse
71 prognosis (3), has become an important therapeutic target (4), and its frequent presence at relapse
72 suggests that *FLT3*-ITD AML-initiating cells are key targets for long-lasting remission.

73 FLT3 tyrosine kinase inhibitors (FLT3-TKI) have been developed as ATP-competitive inhibitors. First-
74 generation FLT3-TKI have a slight single agent activity but showed clinical efficacy in randomized placebo-
75 controlled trials in association with an intensive chemotherapy (5,6). Second-generation FLT3-TKI, such as
76 Quizartinib and Gilteritinib, are more specific for FLT3 and have recently shown significant activity as a
77 single agent in relapsed/refractory (R/R) AML, with composite complete remissions ranging from 45% to
78 55%, associated with prolongation of overall survival (OS) compared with other standards of care (7,8).
79 Quizartinib is a FLT3-TKI that has been specifically optimized pharmacodynamically and pharmacokinetically
80 to inhibit the *FLT3*-ITD mutant protein. This drug induces clearance of peripheral blasts through induction
81 of apoptosis, whereas cell cycle arrest and terminal myeloid differentiation have been shown in bone
82 marrow blasts (9). Gilteritinib is a dual FLT3 and AXL inhibitor with *in vitro* activity against both *FLT3*-ITD
83 and tyrosine kinase domain (TKD) mutations. Gilteritinib allows sustained FLT3 inhibition; response rates
84 are close to those observed with quizartinib, but complete remissions are much more frequent. Gilteritinib
85 induces two distinct bone marrow responses in *FLT3*-mutated AML, with and without differentiation of
86 leukemic cells (10).

87 The other RTK inhibited by Gilteritinib, AXL, is a RTK that is activated by homodimerization upon binding of
88 its ligand growth arrest-specific 6 (GAS6). The GAS6/AXL pathway contributes to cell growth, survival,

89 chemotaxis, apoptotic body clearance and immunity in physiological conditions (11). Moreover, AXL is over-
90 expressed in a broad range of cancers and is regularly associated with a poor prognosis. In AML, AXL and
91 GAS6 levels of expression have been related to poor outcomes where paracrine AXL activation has been
92 shown to induce AML resistance to various therapies (12,13). Finally, we previously reported that several
93 microenvironment-related mechanisms, such as hypoxia and STAT5-activating cytokines, converge to
94 enhance AXL expression and activation, thereby sustaining pro-survival signals that selectively protect *FLT3*-
95 ITD AML cells in the hematopoietic niche (14).

96 Here, we were interested in testing the efficacy of Gilteritinib, as a dual FLT3 and AXL inhibitor, in AML cells
97 within their specific microenvironment. We show that Gilteritinib retains efficacy *in vitro* in *FLT3*-ITD AML
98 despite bone marrow stromal cells (BMSC) coculture and hypoxia compared with Quizartinib. Moreover, *ex*
99 *vivo* primary AML blasts display same profile of response and in various AML mouse models, we show that
100 Gilteritinib is particularly relevant in *FLT3*-ITD AML with high allelic ratio. Finally, we also studied toxicity
101 profile of Gilteritinib and Quizartinib on normal murine hematopoiesis in two mice strains.

102

103 **Materials and Methods**

104

105 **Cell culture**

106 MV4-11, MOLM-13 and MOLM-14 are 3 AML cell lines. MV4-11 has a 48, XY, t(4;11)(q21;q23), +8, +19
107 karyotype with a 30 bp *FLT3*-ITD mutation. MOLM-13 has the following karyotype 51(48-52)<2n>XY, +8, +8,
108 +8, +13, del(8)(p1?p2?), ins(11;9)(q23;p22p23) carrying *KMT2A*-*MLL2* mutations and a 21 bp *FLT3*-ITD
109 mutation; finally, MOLM-14 has the following karyotype -49(46-50)<2n>XY, +6, +8, +13,
110 der(2)t(1;2)(q31;q35), ins(11;9)(q23;p22p23), del(14)(q23q32.3), del(16)(q11.2q13.1) including also *KMT2A*
111 rearrangement and a 21 bp *FLT3*-ITD mutation. All AML and bone marrow stromal cell lines (MV4-11,
112 MOLM-13, MOLM-14, MS5) were cultured in α MEM medium, supplemented with 10% fetal calf serum
113 (FCS), 2 mM L-glutamine, 50 U/mL penicillin, and 50 μ g/mL streptomycin. Hypoxia at 1% was induced by
114 incubating cells in a specific oxygen (O₂) chamber (BioSpherix). Stromal cells were implanted for 3 days to
115 reach 80% confluence, then culture medium was removed, and leukemic cells were added to the flask. Co-
116 culture cells were incubated either in a specific O₂ chamber for 1% O₂ hypoxic culture or in an incubator at
117 5% CO₂ for normoxic culture. Cells were cultured with vehicle or gilteritinib (ASP2215; kindly provided by
118 Astellas Pharma) or quizartinib (AC220; LC Laboratories), both dissolved in dimethyl sulfoxide (DMSO).

119

120 **Cell proliferation assay**

121 Cell proliferation assays were performed using MTS tetrazolium (Cell Titer96 Aqueous; Promega). Cells (10⁵)
122 were plated and incubated in quadruplicate into microtiter-plate wells in 100 μ L of culture medium with
123 various doses of gilteritinib. For the analyses, plates were incubated for 3 h with 20 μ L of MTS and read in a
124 microplate auto reader (Imark Biorad) at 490 nm. Results are expressed as the mean optical density of the
125 four-well set for each gilteritinib dose. All experiments were repeated at least three times during the
126 following 4 days. The 50% inhibiting concentrations (IC₅₀) were determined by linear regression analysis.

127

128 **Western blot analysis**

129 Protein lysates were prepared with cell lysis buffer 10× (Cell Signaling Technology) and protein
130 concentrations were determined by BCATM protein assay (Pierce). Equal amounts of proteins were resolved
131 on 12% polyacrylamide gel and transferred to a nitrocellulose or PVDF membrane (TurboBlot; Biorad). After
132 membrane saturation, the blots were incubated with the respective antibody followed by an anti-rabbit or
133 anti-mouse peroxidase-conjugated secondary IgG antibody (Table S1) and revealed with an enhanced
134 chemiluminescence detection method (Western Lightning Chemiluminescence Reagent Plus; Perkin Elmer).
135 Quantification of protein expression was performed by densitometry using Image Lab software (Biorad).
136 Phosphorylation of protein expression was normalized using total protein expression by calculating the
137 ratio of the phosphorylated protein level to their total protein level counterparts. The ratio of the control
138 condition was normalized to 1.

139

140 **Flow cytometric analysis**

141 Cell apoptosis was assessed using an APC-conjugated Annexin V labeling detection kit coupled to flow
142 cytometry and BD FACSDIVATM software (BD Bioscience). For normal hematopoiesis analyses, BM cells were
143 obtained by crushing tibias, femurs and hip with a pestle and mortar. Filtered single cell suspensions were
144 incubated in Fc block and then stained with antibodies. For NSG analyses, following incubation with Fc
145 block, unfractionated BM cell suspensions were stained with lineage markers containing biotin-conjugated
146 anti-CD4, anti-CD5, anti-CD8a, anti-CD11b, anti-B220, anti-Gr-1 and anti-Ter119 antibodies together with
147 APC-conjugated anti-c-Kit. When applicable, to distinguish hCD45 AML xenotransplant cells from mCD45
148 recipient mice cells, FITC-conjugated anti-hCD45 and APC-Cy7-conjugated anti-mCD45 antibodies were
149 included in the antibody cocktail described above. For C57BL/6J analysis, following incubation with Fc
150 block, unfractionated BM cell suspensions were stained with lineage markers containing biotin-conjugated
151 anti-CD4, anti-CD5, anti-CD8a, anti-CD11b, anti-B220, anti-Gr-1 and anti-Ter119 antibodies together with
152 APC-conjugated anti-c-Kit, BV421-conjugated anti-Sca-1, AF700-conjugated anti-CD48, PE-Cy7-conjugated
153 anti-CD150, FITC-conjugated anti-CD34 and PE-conjugated anti-CD135. Antibodies clones are detailed Table
154 S2. Flow cytometry analyses were performed using a LSR Fortessa (BD). Analysis was done using FlowJo v.X
155 (BD).

156

157 **Animal models for *in vivo* studies with subcutaneous xenografts and bioluminescent imaging**

158 NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ immunodeficient mice (NSG) were bred at the University of Bordeaux's
159 animal facility for experiments validated by the French ministry (authorization no. 00048.2). Female
160 animals were included in protocols at 8 weeks old and were monitored weekly for body weight. For
161 subcutaneous xenografts, prior to implantation, cells were resuspended in 25% Matrigel (BD Biosciences)
162 and 75% culture medium without FCS. Female NSG mice received injections into their hind flanks with 1.75
163 $\times 10^6$ cells. Tumor volume was determined three times per week with a caliper and calculated by the
164 formula $(L^2 * L) / 2$. When tumors reached 400–500 mm³, treatment started with quizartinib (5 mg/kg/day
165 body weight) or gilteritinib (5, 10, and 30 mg/kg/day body weight) by daily oral gavage. For MV4-11
166 hematopoietic engraftment, NSG mice were conditioned with intraperitoneal injections of busulfan (Pierre
167 Fabre) at 20 mg/kg/day for 2 days, and then injected intravenously with 10^6 MV4-11 luciferase-transduced
168 cells (10^6 cells/100 μ L) at day 0. At day 7, engraftment was analyzed by whole bioluminescent imaging (BLI)
169 before treating mice with vehicle or quizartinib (5 mg/kg/day body weight) or gilteritinib (30 mg/kg/day
170 body weight) by daily oral gavage for 7 days. At day 14 and day 20, mice were injected intraperitoneally
171 with firefly luciferase substrate D-luciferin (150 mg/kg) before imaging using a photon bioimager and
172 M3Vision software (Biospace Lab).

173

174 **Primary AML patient samples and patient-derived xenografts cells generation**

175 Patients at the University Hospital of Bordeaux gave written informed consent for the use of biological
176 samples for research, in accordance with the Declaration of Helsinki. AML cells were from a 57-year-old
177 woman diagnosed with a *de novo* AML 4 in FAB classification with normal karyotype *FLT3*-ITD with an
178 ITD/wild type ratio at 0.82 at diagnosis and a *NPM1* mutation. Flow cytometry profile was CD34⁺, HLA-DR⁺,
179 CD56⁻, CD13⁺, CD33⁺, CD117⁺, MPO⁺, CD7⁻ and CD19⁻. Patient-derived xenografts (PDX) were generated by
180 retro-orbital injections of 10^6 viable primary AML cells in 8-week-old female NSG mice, previously
181 conditioned over 2 days by intraperitoneal injections of busulfan (Pierre Fabre; 20 mg/kg/injection/day).
182 Bone marrow engraftment of primary human AML cells in each mouse was followed monthly by

183 intrafemoral aspiration and flow cytometry analyses. When levels reached at least 10% human AML cells
184 (CD33⁺CD45⁺; see Table S2 for the antibodies used), the mice were sacrificed and bone marrow cells were
185 collected, pooled, and cryopreserved (Cryostor CS10; Stem Cell Technologies). The *FLT3* status of human
186 AML cells (hereafter AML PDX cells) in bone marrow was assessed as described previously (15). Two
187 batches of AML PDX cells were generated. The first batch was obtained from two mice sacrificed 6 weeks
188 post-transplantation with an ITD length at 45 bp and an *FLT3*-ITD/wild-type ratio of 0.44 and *FLT3*-ITD/*FLT3*
189 total ratio of 0.30, which demonstrated the presence of *FLT3*-ITD and wild-type AML clones. The second
190 batch was obtained from five mice sacrificed 12 weeks post-transplantation. All human cells in this second
191 batch carried just a *FLT3*-ITD of 45 bp, which demonstrated a loss of heterozygosity (LOH).

192

193 **Animal models for *in vivo* studies from AML PDX cells transplantation**

194 AML PDX cells were injected by retro-orbital route in nonconditioned 8-week-old female NSG mice (10⁶
195 viable PDX cells/mouse). After AML PDX engraftment (> 1% of human cells in bone marrow), 4–5
196 mice/group were treated with vehicle (10% DMSO in water) or quizartinib (5 mg/kg/day body weight) or
197 gilteritinib (30 mg/kg/day body weight) by daily oral gavage for 2 weeks. Mice were sacrificed three days
198 after the last gavage and BM collected from the whole rear limb (hip, femur and tibia). AML PDX cells and
199 murine normal hematopoiesis were analyzed by flow cytometry as described previously (16).

200

201 **Animal models for *in vivo* studies in normal murine hematopoiesis**

202 For both NSG and C57Bl/J model, 5 mice/group were treated with vehicle (10% DMSO in water) or
203 quizartinib (5 mg/kg/day body weight) or gilteritinib (30 mg/kg/day body weight) by daily oral gavage for 2
204 weeks. Animals were either sacrificed for BM analysis 3 days post-treatment or kept alive to assess
205 complete blood count over time for a month. Complete Blood count were measured using a Scil Vet ABC
206 Plus (Horiba). Colony forming cells (CFC) assays : CFC assays were performed using MethoCultTM M3434
207 (STEMCELL Technologies). Two replicates were used per group in each experiment. Colonies were tallied at
208 day 12.

209

210 **Animal models for *in vivo* studies from AML PDX cells transplantation in secondary recipient**

211 Live AML PDX cells (10^6 DAPI-negative cells) of each mouse recipient were collected and transplanted for
212 secondary xenografts in nonconditioned 8-week-old female NSG mice. At 9–10 weeks post-transplantation,
213 the mice were sacrificed and AML PDX cells and murine normal hematopoiesis were analyzed by flow
214 cytometry as described previously (16).

215

216 **Statistical analyses**

217 All analyses were performed using GraphPad Prism software. Unless otherwise indicated, data are
218 presented as the mean \pm standard error of the mean. Statistical significance was calculated using the
219 Student's t-test unless otherwise stated. Differences with p values < 0.05 were considered statistically
220 significant with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, nonsignificant.

221

222 Results

223

224 **Gilteritinib exerts antileukemic effects and inhibits AXL despite stromal cells coculture**

225 We first validated the inhibitory effect of increasing doses of gilteritinib on three human *FLT3*-ITD
226 AML cell lines also expressing AXL. The drug inhibited proliferation of the three cell lines in a dose-
227 dependent manner with IC_{50} values at 3.3 ± 0.6 nM for MV4-11, 19.0 ± 3.2 nM for MOLM-13, and 25.0 ± 1.0
228 nM for MOLM-14 (Figure 1A). Additionally, gilteritinib induced a pro-apoptotic effect, as shown by an
229 increased frequency of annexin V⁺ cells: $38.9 \pm 0.8\%$ for MV4-11, $54.9 \pm 2.0\%$ for MOLM-13, and $90.2 \pm 0.8\%$
230 for MOLM-14 at 90 nM for 48 h (Figure 1B). Similar results were obtained at 72 h in all conditions tested
231 (data not shown). These anti-proliferative and pro-apoptotic effects were correlated with decreased
232 Y₅₉₁FLT3 and Y₇₇₉AXL phosphorylation as well as decreased activation of its downstream signaling. Indeed,
233 densitometry showed dose-dependent decrease in Y₇₇₉AXL phosphorylation in three AML cell lines (1 vs
234 0.3; 0.1 ; 0.2; 0.0 (MV4-11); 1 vs 1; 0.7; 0.3; 0.2 (MOLM-13); and 1 vs 0.5; 0.3; 0.2; 0.1 (MOLM-14), for
235 treatment with Gilteritinib at 5 nM, 20 nM, 50 nM and 100 nM, respectively, compared to vehicle
236 treatment. The decrease in FLT3 phosphorylation was also confirmed (1 versus 0.7; 0.5 ; 0.6; 0.4 (MV4-11);
237 1 versus 0.7; 0.4; 0.6; 0.4 (MOLM-13); and 1 versus 0.6; 0.4; 0.4; 0.3 (MOLM-14), for treatment with
238 Gilteritinib at 5 nM, 20 nM, 50 nM and 100 nM, respectively, compared to vehicle treatment. Finally, data
239 quantifications in figure 1C, also showed a dose-dependent decrease in phosphorylation levels for
240 pY₆₉₄STAT5, pSer₄₇₃AKT, and pT₂₀₂/Y₂₀₄ERK for all 3 cell lines.

241

242 **Antileukemic effects of gilteritinib in hypoxia and with stromal cells coculture compared with quizartinib**

243 The role of AXL in resistance to FLT3 inhibition has been demonstrated previously (13,14). We were
244 interested in whether dual FLT3/AXL inhibition provided by Gilteritinib could be more relevant than single
245 FLT3 inhibition by a highly selective drug such as Quizartinib, particularly in the context of the
246 hematopoietic niche. First, we determined that 3 nM Quizartinib and 100 nM Gilteritinib for 48h induced
247 close levels of apoptosis in normoxia and without stromal cells for various *FLT3*-ITD cell lines. This strategy
248 allowed us to compare the specific effect of such doses considering culture conditions mimicking the bone

249 marrow environment. In the absence of BMSC, Gilteritinib and Quizartinib induced quite similar apoptosis
250 in the three *FLT3*-ITD AML cell lines in normoxia or in hypoxia at 1% O₂ (Figure 2A left panel). We previously
251 demonstrated that without BMSC, the level of AXL expression in AML and the level of autocrine secretion
252 of its ligand GAS6 are very low. In contrast, and as demonstrated in a previous work (14), we observed an
253 upregulation of AXL in hypoxia and in BMSC co-culture condition (Figure 2B). Finally, we observed a
254 significant increase in apoptosis induced by Gilteritinib in hypoxia with BMSC compared with normoxia:
255 17.7 ± 5.3% *versus* 3.4 ± 2.3% (MV4-11); 15.6 ± 7.9% *versus* 3.7 ± 1.2 (MOLM-13); and 44.9 ± 5.4% *versus*
256 15.8 ± 1.7% (MOLM-14), respectively (Figure 2A right panel).

257 Densitometry exhibited a strong dose-dependent inhibition of Y₇₇₉ AXL phosphorylation by
258 Gilteritinib compared with Quizartinib, particularly in culture conditions associating hypoxia and stromal
259 cells (Figure 2B). However, this decrease of Y₇₇₉ AXL phosphorylation was not correlated with a strong
260 inhibition of ERK and AKT which remained similar for Quizartinib and Gilteritinib suggesting that MAPK and
261 PI3K pathways were mainly dependent of *FLT3* signaling (Figure 2B). Next, we investigated whether such
262 effects were observed *ex vivo* in primary AML blasts. Results are shown in Figure 2C and patients are
263 described in Table S3. These data showed that Gilteritinib 100 nM allows a trend (p=0.06) to induce a
264 higher apoptotic response in AML blast in co-culture as compared to Quizartinib 3 nM. Taken together,
265 these results indicate that Gilteritinib is more efficient in conditions mimicking the hematopoietic niche,
266 suggesting that it could overcome protective mechanisms mediated by the bone marrow
267 microenvironment *in vivo*.

268

269 **Antileukemic effects of gilteritinib compared with quizartinib in mouse models xenografted with MV4-11**

270 Encouraged by the *in vitro* data suggesting a therapeutic benefit of dual *FLT3*/*AXL* inhibition in the
271 bone marrow microenvironment, we were interested in whether Gilteritinib could be more effective than
272 Quizartinib *in vivo*. The objective was to specifically examine the role of the bone marrow
273 microenvironment in the efficacy of both tyrosine kinase inhibitors, so we used 2 settings: one excluding
274 and one including BM microenvironment. In this context, the subcutaneous model appeared relevant to
275 study *in vivo* the efficacy of TKIs outside the bone marrow microenvironment. As previously performed *in*

276 *vitro*, we determined dosing regimens in mice for Quizartinib and Gilteritinib that induced close levels of
277 response in a subcutaneous model of *FLT3*-ITD AML cell line. We subcutaneously xenografted MV4-11 cells
278 into immunodeficient NSG mice. When the tumor size reached 400–500 mm³, the mice were treated by
279 Gilteritinib five times per week for 2 weeks with 5 mg/kg/day, 10 mg/kg/day, and 30 mg/kg/day, or 5
280 mg/kg/day of Quizartinib, which was previously determined to be an effective dose *in vivo* (14). According
281 to guidelines for dose conversion between animals and humans (17) and the quantitative determination of
282 quizartinib in mouse plasma (18), we expected that 5 mg/kg/day corresponds to a dosing regimen ranging
283 from 40–50 mg/day in humans (19,20). At day 28, the median tumor volume was increased to 777.1 mm³
284 with 5 mg/kg/day of gilteritinib, whereas it decreased to 84.2 mm³ with 10 mg/kg/day of gilteritinib. Mice
285 were in complete remission at day 28 with Quizartinib 5 mg/kg/day and Gilteritinib 30 mg/kg/day with the
286 same response profile, and at day 40 with Gilteritinib 10 mg/kg/day. Treatment by *FLT3*-TKI was
287 discontinued when mice were in complete remission, except for mice in the Gilteritinib 5 mg/kg/day arm,
288 which were sacrificed at day 28 for ethical reasons (Figure 3A). Finally, median time to relapse was 13 days
289 for Quizartinib 5 mg/kg/day arm and 14 days for Gilteritinib 30 mg/kg/day arm (*p*=NS) whereas it was at 2
290 days for Gilteritinib 10 mg/kg/day arm (*p*<0.001 compared with the two other arms) (Figure 3B). Based on
291 the quantitative determination of the gilteritinib in mouse plasma (21), we expected that 30 mg/kg/day
292 corresponds to a dosing regimen at 120 to 130 mg/day in humans (22). Finally, both dosing regimens
293 coincided with clinically relevant posology used as single agents in clinical trials.

294 This strategy allowed us to compare the specific effect of such doses considering engraftment in
295 the bone marrow environment in the next experiment. We first used *FLT3*-ITD MV4-11 cells that were
296 xenografted by vein injection. Leukemic cell engraftment was analyzed by bioluminescence imaging (BLI) as
297 described previously (14). At day 7, one group of the mouse cohort was treated by daily oral gavage for 14
298 days with Quizartinib (5 mg/kg/day), one group by Gilteritinib (30 mg/kg/day), and a control group with
299 vehicle. Response to treatment was assessed by BLI at day 14, and then at day 20 (Figure 3C). At day 14, a
300 significant decrease in total bioluminescence signal was observed in response to Quizartinib compared with
301 the control group (4.3×10^7 Ph/s/sr *versus* 1.5×10^6 Ph/s/sr, *p* < 0.001) and in response to Gilteritinib
302 compared with the control group (4.3×10^7 Ph/s/sr *versus* 0.8×10^6 Ph/s/sr, *p* < 0.001). This leukemic

303 burden decrease was stronger at day 14 (0.8×10^6 Ph/s/sr versus 1.5×10^6 Ph/s/sr, $p < 0.05$) and at day 20
304 (0.5×10^6 Ph/s/sr versus 1.3×10^6 Ph/s/sr, $p < 0.05$) with Gilteritinib compared with Quizartinib,
305 respectively (Figure 3C).

306

307 **Antileukemic effects of Gilteritinib and Quizartinib in *FLT3*-ITD AML patient-derived xenografts**

308 Encouraged by these *in vivo* results with the *FLT3*-ITD AML cell line MV4-11, we explored the effects
309 of these two *FLT3*-TKI *in vivo* on two different batches of AML-PDX cells generated from a *FLT3*-ITD AML
310 patient using the process presented in Figure 4A. The first batch contained *FLT3*-ITD and wild-type AML
311 cells with an *ITD*/wild-type ratio of 0.44. The second batch contained *FLT3*-ITD AML cells with LOH. We
312 transplanted AML PDX cells of these two batches into NSG mice and monitored engraftment in bone
313 marrow using flow cytometry (CD45/CD33 double staining). Then, mice were treated by daily oral gavage
314 with Quizartinib (5 mg/kg/day) and Gilteritinib (30 mg/kg/day) for 2 weeks. Response to treatment was
315 assessed by flow cytometric analysis of human CD45⁺/CD33⁺ blasts from crushed femurs, tibias, and hips
316 (Figure 4B). Analysis of the results based on molecular characterization of the transplanted batches
317 confirmed that mean percentage of human CD45⁺/CD33⁺ blast cells in the bone marrow at week 9 post-
318 transplantation was significantly higher in Quizartinib-treated mice (27.8%±5.8) than in Gilteritinib-treated
319 mice (15.4%±2.5) in *FLT3*-ITD AML PDX with LOH (Figure 4C). Conversely, mean percentage of human
320 CD45⁺/CD33⁺ blast cells in the bone marrow at week 9 post-transplantation was similar in Quizartinib-
321 treated mice and in Gilteritinib-treated mice in *FLT3*-ITD AML PDX with an *ITD*/wild-type ratio of 0.44
322 (Figure 4D). These results suggested that gilteritinib displayed a higher level of efficacy in BM
323 microenvironment for AML with high *FLT3*-ITD/wt ratio.

324

325 **Gilteritinib spared normal murine hematopoiesis in a BM microenvironment impaired by AML**

326 After demonstrating the potential for dual *FLT3*/AXL inhibition, we were interested in the tolerance
327 of normal murine hematopoiesis to Gilteritinib treatment compared with Quizartinib as it also impacts C-Kit
328 signaling. Three days post-*FLT3*-TKI treatment, there were no differences in BM cellularity between vehicle-
329 treated mice, quizartinib-treated mice, and gilteritinib-treated mice in a non-transplanted NSG group

330 (Figure 5A, left panel). In contrast, in AML-PDX NSG group, murine leukocytes cellularity was significantly
331 higher in gilteritinib-treated mice (median 89.3×10^6 , IQR 83.7-95.3) than in quizartinib-treated mice
332 (median 73.0×10^6 , IQR 45.1-78.6) (Figure 5A, right panel). Finally, comparing across transplanted and non-
333 transplanted groups, we showed that murine BM cellularity was returned to normal only with Gilteritinib.
334 When separating murine leukocytes between differentiated precursor cells defined as $mCD45^+ Lin^+$ cells
335 (Figure 5B) and a broader immature population defined as $mCD45^+ Lin^-$ cells (Figure 5C), the same
336 conclusions can be drawn. We next decided to investigate the more primitive compartments (Figure S3A)
337 such as HSPC using $mCD45^+ Lin^- c-Kit^{High}$ immunophenotyping approach: there were here some differences
338 between vehicle-treated mice, quizartinib-treated mice and gilteritinib-treated mice in the non-
339 transplanted NSG group suggesting a slight impairment of this compartment induced by FLT3-TKI,
340 particularly Gilteritinib (median 5.7×10^6 [IQR 5.3-6.3] vs 4.6×10^6 [IQR 4.4-5.3] vs 4.2×10^6 [IQR 3.9-4.4] in
341 Vehicle, Quizartinib and Gilteritinib, in non-transplanted NSG, respectively). However, the absolute
342 numbers of murine $mCD45^+ Lin^- c-Kit^{High}$ cells were similar in both Gilteritinib-treated mice (median 3.5×10^6
343 [IQR 3.3-4.6]) and Quizartinib-treated mice (median 3.2×10^6 [IQR 1.8-4.3]) in AML-PDX NSG group. Finally,
344 as observed for mature compartments, Gilteritinib was able to maintain the level of $mCD45^+ Lin^- c-Kit^{High}$
345 cells in AML-PDX NSG group close to the one observed in Gilteritinib-treated non-transplanted NSG group
346 whereas Quizartinib did not (Figure 5D). It should nevertheless be noted that outside the leukemic context,
347 Gilteritinib impairs normal primitive murine hematopoiesis more than Quizartinib (Figure 5C and 5D, left
348 panels). Taken together, these data could suggest either a better efficacy of Gilteritinib in BM clearance of
349 AML blasts, leading to a better murine hematopoiesis, or a toxicity of Quizartinib on normal murine
350 hematopoiesis specifically in the context of AML, since this point is not observed in non-transplanted NSG
351 mice. To discriminate between these two hypotheses, we analyzed murine hematopoiesis according to BM
352 clearance as previously shown in Figure 4C and 4D. In "LOH" group, BM clearance was higher with
353 Gilteritinib, whereas in "ratio 0.44", BM clearance was the same with both treatments. In both *FLT3*-ITD-
354 AML-PDX group "LOH" and "ratio 0.44", independently of AML blasts clearance, murine leukocyte numbers
355 were higher upon Gilteritinib treatment compared with Quizartinib treatment (Figure 5E). This rules in

356 favour of a lower toxicity for Gilteritinib for maintaining normal murine hematopoiesis in a BM
357 microenvironnement impaired by AML.

358 In front of these results, we next assessed HSPC functions. First, we performed CFC assay at
359 sacrifice of non-transplanted NSG mice and found that none of the FLT3-TKI impaired progenitors' ability to
360 generate colonies (Figure 5F). Then, we analyzed Complete Blood Count (CBC) at day 4, day 18 and day 31
361 post-TKI treatment in a non-transplanted mice group. We did not find any clinically relevant effects of
362 either TKI, Quizartinib or Gilteritinib, supporting the fact that these treatments are well tolerated at these
363 doses on a steady-state hematopoiesis whether on WBC, Red blood cells, platelets, lymphocytes,
364 monocytes, or granulocytes (Figure S1). To confirm our conclusions, we performed a new set of
365 experiments on C57Bl/6J mice. During treatment, complete blood count was followed and did not show
366 any differences between the 3 groups (Figure S2). As observed in NSG counterparts, three days post-FLT3-
367 TKI treatment, there were no significant differences between vehicle-treated mice, quizartinib-treated mice
368 and gilteritinib-treated mice, for the following cell subsets : Lin⁻ cells (Figure 5G), Lin⁻ Sca1⁺ c-Kit^{High} cells
369 (Figure 5H) and HSC immunophenotypically defined as LSK CD48⁻ CD150⁺ CD135⁻ CD34⁻ (Figure 5I/S3B). We
370 also analyzed CFU-GEMM, -GM, -G, -M and Red/Mk (Figure 5J) without noticing any significant differences.

371 Taken together, these data support the fact that Gilteritinib and Quizartinib display close toxicity
372 profile on normal murine hematopoiesis at steady state, even if Gilteritinib display a slight impairment of
373 primitive compartment without impact on CBC. Conversely, Quizartinib display higher toxicity on murine
374 hematopoiesis in BM impaired by AML.

375

376 **Effects of gilteritinib and quizartinib on leukemic initiating cell compartment in secondary AML PDX**
377 **recipient**

378 To further explore the ability of both FLT3-TKI to target leukemic initiating cells in their
379 microenvironment, we performed secondary transplants from bone marrow of Gilteritinib-treated and
380 Quizartinib-treated AML PDX cells (10⁶ AML PDX alive cells/secondary recipient) as described in Figure 4B.
381 Once again, analysis of the results (Figure S4) based on molecular characterization of the engrafted batch
382 revealed that gilteritinib was more effective than quizartinib in controlling leukemic stem cell (LSC)

383 engraftment and leukemic burden in *FLT3*-ITD AML PDX with LOH (Figure 6A) compared with *FLT3*-ITD AML
384 PDX with an ITD/wild-type ratio at 0.44 (Figure 6B), in which Quizartinib and Gilteritinib displayed the same
385 levels of efficacy.

386

387 **Discussion**

388

389 Here, we demonstrated that dual AXL and FLT3 inhibition should target *FLT3*-ITD leukemic cells
390 protected from cytotoxic treatments by various mechanisms including low O₂ concentration, BMSC, and
391 soluble factors in the bone marrow microenvironment. AXL is ectopically- or over-expressed in a wide
392 variety of cancers and has always been associated with a poor prognosis (23). We have reported resistance
393 mechanisms involving AXL in chronic myeloid leukemia (24). In AML, AXL and GAS6 levels of expression
394 have been related to poor outcomes (25,26). Paracrine AXL activation has been shown to induce AML
395 resistance to conventional chemotherapies but also to FLT3-targeted therapy (12,13,27,28). We previously
396 demonstrated that AXL was particularly relevant in the setting of the hematopoietic niche (14), suggesting
397 that Gilteritinib could be a candidate to improve *FLT3*-ITD AML-initiating cell eradication.

398 We confirmed that Gilteritinib induced dose-dependent antiproliferative and pro-apoptotic effects
399 associated with an inhibition of FLT3 activation and its downstream signaling (21). We demonstrated the
400 specific interest of Gilteritinib in the setting of hypoxia and BMSC coculture, linked to a better efficacy
401 compared with Quizartinib, another FLT3-TKI much more specific of FLT3. Gilteritinib also provided better
402 response to treatment in bioluminescent imaging with a mouse model xenografted with a *FLT3*-ITD AML
403 cell line. Then, in a *FLT3*-ITD AML PDX model, we found that Gilteritinib as single agent was more effective
404 in controlling *FLT3*-ITD AML with a high allelic ratio, whereas its efficacy was similar to that of Quizartinib
405 for AML with *FLT3*-ITD and *FLT3* wild-type clones. Finally, by performing a secondary xenograft, we
406 reproduced identical results but neither Quizartinib nor Gilteritinib fully eradicated leukemic initiating cells.
407 Nevertheless, Gilteritinib-treated AML PDX displayed extended time in terms of leukemic burden,
408 specifically in the *FLT3*-ITD AML with LOH batch. We could speculate that such efficacy by continuous
409 treatment longer than 2 weeks could perhaps enable deeper impairment of *FLT3*-ITD AML clone. Finally, we
410 could also speculate that other kinases inhibited by Gilteritinib could also play a role in the bone marrow
411 microenvironment such as LTK or ALK (21). Indeed, effects of mesenchymal stromal cells and endothelial
412 cells on AML cells have been investigated through contact factors and cytokine-mediated crosstalk. Several
413 soluble mediators are known to be linked with remodeling of the HSC niche into a leukemic-permissive

414 niche such as angiogenic growth factors like VEGF, Ang-1, pro-inflammatory cytokines such as IL-6, homing-
415 disrupting cytokines such as CXCL12. Other studies also detected variation according to osteoblastic or
416 adipocytic MSC differentiation, but also immune cells secreting FLT3-ligand or IL-1 (29).

417 Using two *FLT3*-ITD AML PDX models, we showed that quizartinib was as effective as gilteritinib
418 when PDX displayed both *FLT3*-ITD and wild-type clones. These experiments reproduced outcomes of
419 phase 2 clinical trials in which FLT3-TKI as single agent allowed 12% and 45% of overall response in *FLT3*
420 wild-type AML for gilteritinib (30) and quizartinib (31), respectively. Quizartinib is known to inhibit KIT
421 phosphorylation, leading to an impaired hematopoiesis (7). If Gilteritinib does not inhibit KIT, its use in
422 routine clinical practice unveils also a certain impairment of normal hematopoiesis, independently of its
423 ability to induce blast differentiation (9,10). Here, we provide some data regarding murine hematopoiesis,
424 demonstrating that Gilteritinib and Quizartinib display close toxicity profile on normal murine
425 hematopoiesis at steady state without impact on CBC and potentially different toxicity profiles on
426 hematopoiesis impaired by AML involvement.

427 These different endpoints raise an issue with clinical implications. In this work, Quizartinib was
428 equally effective as Gilteritinib as a single agent in AML carrying both *FLT3*-ITD and *FLT3* wild-type clones.
429 Both FLT3-TKI have been developed in the R/R situation and the phase 3 outcomes showed that Gilteritinib
430 was better for complete remission but just slightly better for overall survival. In a front-line situation, *FLT3*-
431 ITD AML also carries *FLT3* wild-type clones and is treated with intensive chemotherapy, which by itself
432 induces deep toxicity in normal hematopoiesis. Quizartinib could be an effective treatment in this case
433 regardless of its action on normal hematopoiesis. Moreover, priming with Quizartinib has recently been
434 shown to inhibit chemotherapy-induced myelosuppression by inducing a transient quiescence of
435 multipotent progenitors, protecting these cells from chemotherapy (32). In contrast, Gilteritinib as single
436 agent is more effective and less toxic than standard chemotherapy, establishing a new standard of care for
437 management of R/R *FLT3*-mutated AML. Our results suggest that its intrinsic characteristics make it most
438 effective as single agent in high allelic ratio *FLT3*-ITD AML.

439 As single agents, FLT3 TKI such as Gilteritinib or others are unlikely to result in a high cure rate for
440 *FLT3*-ITD AML. Gilteritinib, Quizartinib, or Crenolanib could improve the cure rates for patients with *FLT3*-

441 mutated AML, but only when incorporated into a treatment regimen that includes other chemotherapies or
442 drugs. In that setting, ability of Gilteritinib to inhibit AXL could be a leverage to eradicate *FLT3*-mutated
443 AML clones but could also help to target *FLT3* wild-type AML clones shielded by their microenvironment
444 since AXL resistance to chemotherapy is encountered beyond *FLT3*-ITD AML cells. These data strongly
445 support current clinical trials associating chemotherapy and Gilteritinib.
446

447 **Figure legends**

448

449 **Figure 1: Activity of gilteritinib against *FLT3*-ITD AML cell lines**

450

451 A. Relative proliferation of MV4-11, MOLM-13, and MOLM-14 *FLT3*-ITD AML cell lines incubated in the
452 absence (Vehicle) or with increasing concentration of gilteritinib (as indicated) for 4 days using CellTiter 96
453 AQueous. Condition Vehicle with DMSO 0.1%. The proliferation of the cells treated with the vehicle control
454 was set as 100% (mean \pm standard error of the mean; n = 4).

455

456 B. *FLT3*-ITD AML cell lines were treated in the absence (Vehicle) or with increasing dose of gilteritinib (as
457 indicated) for 48 h. Apoptosis was measured by Annexin V/DAPI staining followed by flow cytometric
458 analysis. Results are presented as the percentage of the Annexin V cells. Condition Vehicle with DMSO
459 0.1%. Mean \pm standard error of the mean; n = 3 with *, p < 0.05; **, p < 0.01; ***, p < 0.001; NS,
460 nonsignificant.

461

462 C. Western blot analysis using indicated antibodies performed on lysates from MV4-11, MOLM-13, and
463 MOLM-14 cell lines. Cells were exposed to gilteritinib at the indicated concentrations for 4 h. Condition 0
464 nM (Vehicle) with DMSO 0.1%. Results shown are representative of three independent experiments with
465 densitometry.

466

467 **Figure 2: Activity of Gilteritinib compared with Quizartinib in vitro and ex vivo in hematopoietic niche**
468 **condition**

469

470 A. Apoptosis assessment of *FLT3*-ITD AML cell lines MV4-11, MOLM-13 and MOLM-14 treated with
471 quizartinib (3 nM) or gilteritinib (100 nM) for 48 h, and cultured without (no stroma, left panel) or with the
472 MS5 stromal cells (stroma, right panel), at 21% (solid bars) or 1% (hatched bars) of O₂ concentration, using
473 flow cytometry. Condition Vehicle with DMSO 0.1%. Mean ± standard error of the mean; n = 3 with *, p <
474 0.05; **, p < 0.01; ***, p < 0.001; NS, nonsignificant.

475

476 B. MOLM-14 cells were incubated with quizartinib (3 nM) or gilteritinib (20 or 100 nM) for 4h and cultivated
477 without (no stroma) or with MS5 stromal cells (stroma) at 21% or 1% of O₂ concentration, as indicated.
478 Total cell lysates of the samples were subjected to western blot analysis with the indicated antibodies.
479 Results shown are representative of two independent experiments. Condition Vehicle with DMSO 0.1%.
480 Results shown are representative of three independent experiments with densitometry : phosphorylation
481 of protein expression was normalized using total protein expression by calculating the ratio of the
482 phosphorylated protein level to their total protein level counterparts. The ratio of the control condition was
483 normalized to 1. Mean of n=3 WB with *, p < 0.05; **, p < 0.01; ***, p < 0.001; NS, nonsignificant.

484

485 C. Primary blasts from 6 *FLT3*-ITD AML patients cultured with the MS5 stromal cells at 20% O₂, were treated
486 without (vehicle) or with Quizartinib (3 nM) or Gilteritinib (20 or 100 nM) for 48 hrs. Cells were then stained
487 with Annexin V before cytometer analysis. Condition Vehicle with DMSO 0.1%. Graph shows the mean ±
488 SEM of results with *, p < 0.05; **, p < 0.01; NS, non-significant.

489

490 **Figure 3: Dual AXL/FLT3 inhibition by gilteritinib exerts *in vivo* a higher pro-apoptotic effect than**
491 **quizartinib in bone marrow microenvironment**

492

493 A. Dose-dependent inhibition of MV4-11 tumor growth by FLT3-TKI. MV4-11 cells were subcutaneously
494 injected into the limbs of NSG mice at day 0 and treated or not with quizartinib (5 mg/kg/day) or at various
495 dosing regimens of gilteritinib (5, 10, and 30 mg/kg/day). Tumor volume was measured at the indicated
496 days (mean \pm standard error of the mean; n = 40) with *, p<0.05; **, p < 0.01; NS, non-significant.

497

498 B. Time to relapse after gilteritinib or quizartinib discontinuation. Tumor growth was monitored at the
499 indicated days (mean \pm standard error of the mean; n = 40).

500

501 C. MV4-11 Luc cells were injected at day 0 (10^6 cells/mouse) in the retro-orbital sinus vein of busulfan-
502 pretreated female mice. Mice were then treated or not with quizartinib (5 mg/kg/day) or gilteritinib (30
503 mg/kg/day) from day 7 to day 20. At day 14 and day 20, animals were analyzed using bioluminescent
504 imaging (n = 30). Statistical analyses were performed using the log-rank test. *, p < 0.05; **, p < 0.01; ***, p
505 < 0.001; NS, non-significant.

506

507 **Figure 4: Anti-leukemic effects of gilteritinib and quizartinib in a *FLT3*-ITD AML patient-derived xenograft**

508

509 A. Patient-derived xenografts (PDX) were generated by retro-orbital injections of 10^6 viable primary AML
510 cells in NSG mice previously conditioned. When levels reached at least 10% human AML cells (CD33⁺CD45⁺),
511 the mice were sacrificed and bone marrow cells were collected, pooled, and cryopreserved. Two batches of
512 AML PDX cells were generated. The engraftment in each mouse was assessed every 10 days by intra-
513 femoral puncture analyzed by flow cytometry to measure hCD45 chimerism. The mice were sacrificed when
514 the engraftment was higher than 10%. LOH: loss of heterozygosity.

515

516 B. AML PDX cells were injected by retro-orbital route in nonconditioned 8-week-old female NSG mice (10^6
517 viable PDX cells/mouse). After AML PDX cells engraftment (> 1% of human cells in bone marrow; 6–7 weeks
518 post-transplantation), the mice were divided in three homogeneous groups and treated with vehicle (water
519 10% DMSO) or Quizartinib (5 mg/kg/day) or Gilteritinib (30 mg/kg/day) by daily oral gavage during 2 weeks.
520 Three days after the last gavage, the mice were sacrificed, analyzed, and 10^6 AML PDX cells were serially
521 transplanted into secondary nonconditioned recipients. The secondary recipients were sacrificed and
522 analyzed 9–10 weeks post-transplantation.

523

524 C. PDX model from a *FLT3*-ITD AML patient was used to test the *in vivo* efficacy of treatment with
525 gilteritinib and quizartinib. The percentage of human CD45⁺/CD33⁺ blast cells in the bone marrow from
526 weeks 8–9 post-transplantation (sacrifice) was determined based on flow cytometric analysis in AML PDX
527 from batch 2 (LOH) at week 7 (before treatment) and week 9 post-transplantation (after treatment). Mean
528 \pm standard error of the mean with *, $p < 0.05$; **, $p < 0.01$; NS, nonsignificant ($n = 10$).

529

530 D. PDX model from a *FLT3*-ITD AML patient was used to test the *in vivo* efficacy of treatment with
531 gilteritinib and quizartinib. The percentage of human CD45⁺/CD33⁺ blast cells in the bone marrow from
532 weeks 8–9 post-transplantation (sacrifice) was determined by flow cytometric analysis in AML PDX from

533 batch 1 (ratio ITD/wt 0.44) at week 7 (before treatment) and week 9 post-transplantation (after treatment).

534 Mean \pm standard error of the mean with *, $p < 0.05$; **, $p < 0.01$; NS, nonsignificant (n = 5).

535

536 **Figure 5: Normal murine hematopoiesis in non-transplanted and FLT3-ITD AML-PDX mice**

537

538 Determination of Murine CD45⁺ cells (A), murine Lin⁺ cells (B), murine Lin⁻ cells (C), and Lin⁺ c-Kit^{High} cells (D)
539 in the bone marrow of NSG mice transplanted or not with AML-PDX cells 3 days post-treatment with
540 vehicle, Quizartinib or Gilteritinib. (E) Murine CD45⁺ cells in bone marrow of NSG mice transplanted with
541 AML-PDX with LOH or with ITD/wt at 0.44, 3 days post-treatment with vehicle, Quizartinib or Gilteritinib. (F)
542 Colony forming unit (CFU) assay performed with 10⁴ BM cells from non-transplanted NSG mice 3 days post-
543 treatment with vehicle, Quizartinib or Gilteritinib. Murine Lin⁻ cells (G), murine Lin⁻ Sca-1⁺ c-Kit^{High} cells (H),
544 and HSC (Lin⁻ c-Kit⁺ Sca-1⁺ CD48⁻ CD150⁺) (I) in the bone marrow of C57Bl/6J mice 3 days post-treatment
545 with vehicle, Quizartinib or Gilteritinib. (J) Colony forming unit (CFU) assay performed with 10⁴ BM cells
546 from C57Bl/6J mice 3 days post-treatment with vehicle, Quizartinib or Gilteritinib. CFU-megakaryocyte
547 (CFU-Mk), CFU-erythroid (CFU-E), Burst Forming Unit (BFU-E), CFU-granulocyte (CFU-G), CFU-
548 monocyte/macrophage (CFU-M), CFU-granulocyte and monocyte/macrophage (CFU-GM), CFU with all 4
549 lineages granulocyte, erythroid, monocyte/macrophage and megakaryocyte (CFU-GEMM). The data are as
550 mean ± standard error of the mean (n = 5 mice per group for non-transplanted mice and n = 15 for AML-
551 PDX transplanted mice) with *, p < 0.05; **, p < 0.01; ***, p < 0.001; NS, non-significant.

552

553 **Figure 6: Antileukemic effects of gilteritinib and quizartinib in a secondary recipient of *FLT3*-ITD AML**
554 **patient-derived xenograft**

555

556 10^6 living human CD45+ cells issued from primary recipients were injected into non-conditioned secondary
557 recipients. Percentage of human CD45+/CD33+ blast cells in the bone marrow 10 weeks post-
558 transplantation, which was determined based on flow cytometric analysis for AML PDX from batch 2 (A)
559 and for AML PDX from batch 1 (B). Mean of five mice per cohort \pm standard error of the mean with *, $p <$
560 0.05; **, $p < 0.01$; ***, $p < 0.001$; NS, nonsignificant.

561

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669 **Conflict of Interest**

670 Pierre-Yves Dumas: Advisory boards: Daiichi-Sankyo, Astellas

671 Arnaud Villacreces: no disclosure

672 Amelie Guitart: no disclosure

673 Layal Massara: no disclosure

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