Concomitant targeting of FLT3 and BTK with CG-806 overcomes FLT3-inhibitor resistance through inhibition of autophagy

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Abstract: 2635

Background

Fms-like tyrosine kinase 3 (FLT3)-targeted therapy represents an important paradigm in the management of patients with highly aggressive *FLT3*-mutated acute myeloid leukemia (AML). However, clinical efficacy is usually transient and followed by emergence of resistance (Borthakur *et al*., 2011; Cortes *et al*., 2013; Zhang *et al*., 2008). Such resistance often results from acquired mutations of TKD, which are frequently identified in the D835, Y842 and F691 residues of the protein (Smith *et al*., 2015; Smith *et al*., 2012; Zhang *et al*., 2014). It was reported that the FLT3-ITD-targeting drug sorafenib can induce autophagy in human myeloid dendritic cells (Lin *et al*., 2013). Induction of autophagy has also been reported to play a crucial role in resistance to BCR-ABL inhibitor imatinib in CML (Hekmatshoar *et al*., 2018). Additionally, inhibition of autophagy can re-sensitize cancer cells to apoptosis induction (Fitzwalter *et al*., 2018; Piya *et al*., 2017), suggesting the possibility that inhibition of autophagy may represent a novel therapeutic strategy for overcoming resistance to FLT3-targeted therapy.

Methods

- Leukemia cell lines and AML patient samples were exposed to the small molecule pan-FLT3/pan-BTK inhibitor CG-806 (Zhang *et al*., 2017, Yu *et al*., 2017) or other indicated compounds in the presence/absence of mesenchymal stem cells (MSCs)/hypoxic conditions *in vitro*. Apoptosis induction was evaluated using FACS by measuring annexin V positivity.
- Cells lysates were collected. Total and phosphorylated levels of the indicated proteins were determined by immunoblotting.
- MOLM14-BTK-KD cells were generated by introducing BTK siRNA (Dharmacon) with the Nucleofection electroporation system (Amaxa) following the manufacturer's instructions.
- For TEM analysis, the treated MOLM14 cells were fixed, Epon 812 embedding and staining followed regular TEM tissue processing. Observed in a JEM 1010 TEM, 80 KV.
- A PDX murine model was established by *i.v.* injection of an AML patient sample (harboring FLT3-ITD plus D835) into NSG mice. The mice started treatment with CG-806 at 100mg/kg dose when reaching 1% engraftment in blood (i.e. Day 27). Engraftment was analyzed using FACS by measuring hCD45$^+$/mCD45$^-$ population. Mouse survival was estimated by the Kaplan–Meier method with log-rank statistics.

Results

Fig. 1. FLT3-inhibitor Resistant Cells and Primary AML Samples Post Sorafenib Therapy Show High Basal Levels of Autophagy

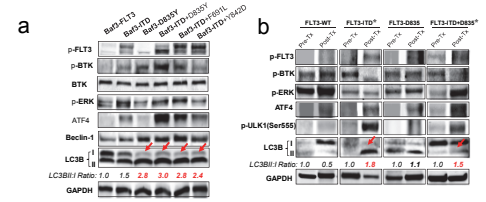


Fig. 1. Baf3 cells, or AML patient samples bearing variety of FLT3 mutations, were collected for determining protein expression with immunoblotting. * These AML patients showed no response after receiving sorafenib administration in Phase I clinical trial.

Fig. 2. Hypoxia and Co-culture with MSCs Upregulate Autophagy and Phosphorylated BTK Level in FLT3-mutated Leukemic Cells

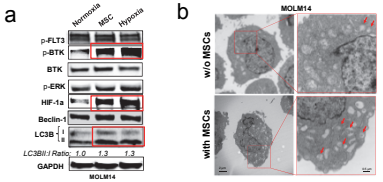


Fig. 2. MOLM14 cells were co-cultured with MSCs or in normoxic/hypoxic conditions for 48 h, The cells were collected for (a) immunoblotting and (b) TEM analyses. Red arrows indicates autophagic vesicles.

Fig. 3. Autophagy Inhibition with Chloroquine (CQ) Enhances Quizartinib-induced Apoptosis and Partially Abrogates MSCs-mediated Protection

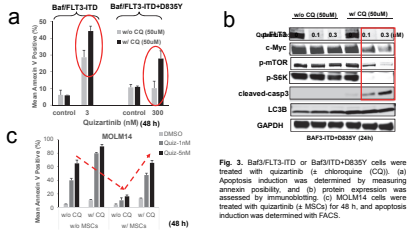


Fig. 3. Baf3/FLT3-ITD or Baf3/ITD+D835Y cells were treated with quizartinib (± chloroquine (CQ)). (a) Apoptosis induction was determined by measuring annexin posibility, and (b) protein expression was assessed by immunoblotting. (c) MOLM14 cells were treated with quizartinib (± MSCs) for 48 h, and apoptosis induction was determined with FACS.

Fig. 4. BTK Inhibition Sensitizes to Quizartinib-induced Leukemic Cell Killing and Abrogates MSCs-mediated Protection of FLT3-mutated Leukemia Cells

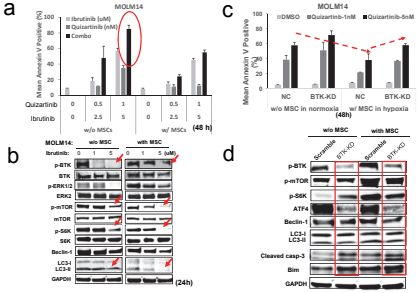


Fig. 4. (a, b) MOLM14 cells or (c, d) MOLM14-scramble/BTK-siRNA-knocking down cells were treated with ibrutinib/quizartinib alone or combo (±MSCs) and apoptosis induction and protein expression were determined with FSCS and immunoblotting.

Fig. 5. The FLT3/BTK Inhibitor CG-806 Abolishes MSCs/hypoxia-mediated Protection and Induces Apoptosis in FLT3-mutated Leukemia Cells

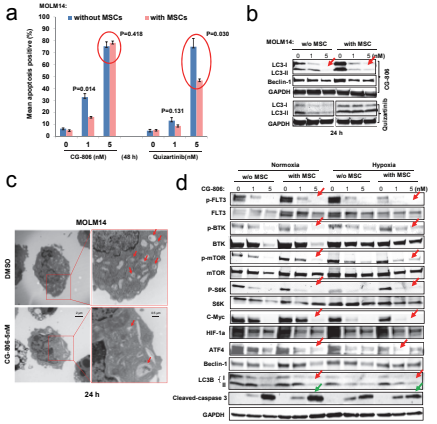


Fig. 5. MOLM14 cells were treated with CG-806/quizartinib (±MSCs), and (a) apoptosis and (b) protein expression were determined with FACS and immunoblotting. (c) Autophagosome was analyzed by TEM. Red arrows indicates autophagic vesicles. (d) MOLM14 cells were treated with CG-806 in normoxic/hypoxic conditions (±MSCs) for 24h. Protein expression was determined with immunoblotting analysis. Red arrows indicated downregulation and green arrows indicated upregulation.

Fig. 6. The FLT3/BTK Inhibitor CG-806 Exerts Efficient Anti-leukemia Activity in FLT3-inhibitor Resistant AML, Represses Autophagy Induction and Extends Mouse Survival in a FLT3-ITD+D835 Bearing PDX Leukemia Model

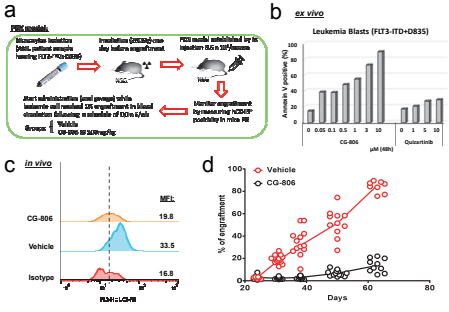
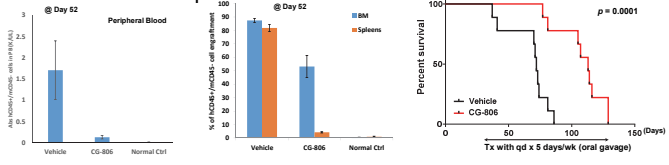
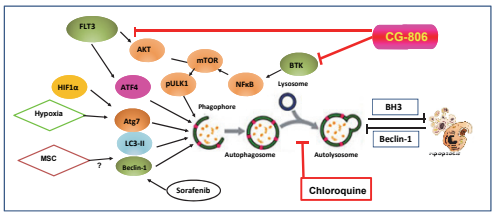


Fig. 6. (a) NSG mice PDX model by xenografting with FLT3-ITD + D835 mutated AML patient sample. (b) Engrafted leukemia cells (hCD45+/mCD45-) were treated with CG-806 or Quizartinib *ex vivo*, and apoptosis was determined with FACS. (c) LC3 levels of engrafted leukemic cells were determined with FACS after CG-806 administration. (d) Leukemia burden (hCD45+mCD45- percentage) of peripheral blood was assessed by FACS. Three mice/group were sacrificed and engraftment (hCD45+mCD45- percentage) was assessed in (e) peripheral blood, (f) bone marrow and spleen by FACS. (g) Mouse survival (n = 7 mice/group) was estimated by the Kaplan–Meier method with log-rank statistics. Median survival are 72 and 113 in Vehicle and CG-806 (100mg/kg), respectively.
PDX, patient-derived xenograft; Tx, treatment.



Summary:



Conclusions

- FLT3-inhibitor resistant (FLT3-ITD plus TKD mutated) cells or FLT3-targeted therapy upregulates spontaneous autophagy levels and accompanies increase of phospho-BTK.
- Autophagy and phospho-BTK increase in the presence of MSCs/hypoxia environment in FLT3-mutated AML.
- Inhibition of autophagy enhances quizartinib-induced pro-apoptotic effects and abrogates MSCs/hypoxia-mediated protection.
- BTK inhibition with ibrutinib or BTK siRNA sensitizes quizartinib-induced anti-leukemia effects in FLT3-mutated AML.
- Pan-FLT3/pan-BTK inhibitor CG-806 abolishes MSCs/hypoxia-mediated protection and triggers apoptosis induction by reducing autophagy and phospho-BTK levels.
- CG-806 triggers profound pro-apoptotic effects and reduces autophagy levels in FLT3-inhibitor resistant AML patient samples in *ex vivo* and *in vivo*.
- CG-806 significantly reduces leukemia burden and extends survival in a FLT3-ITD+D835-bearing PDX human leukemia model.
- Blockade of FLT3/BTK with CG-806 could provide a novel strategy for preventing or overcoming FLT3 inhibitor resistance in FLT3-mutated AML. Phase I trials of CG-806 are in preparation.

Conflict of interest: H. Zhang and W. Rice are employees of Aptose Biosciences; M. Andreeff serves on Aptose Biosciences SAB.