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The dual MEK/FLT3 inhibitor E6201 exerts cytotoxic activity against acute myeloid leukemia cells harboring resistance-conferring FLT3 mutations

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Abstract

FLT3 inhibition has elicited encouraging responses in acute myeloid leukemia (AML) therapy. Unfortunately, unless combined with a bone marrow transplant, disease relapse is frequent. In addition to the acquired point mutations in the FLT3 kinase domain that contribute to FLT3 inhibitor resistance, MEK/ERK signaling is persistently activated in AML cells even when FLT3 phosphorylation is continually suppressed. Thus, concomitant targeting of FLT3 and MAPK may potentially exert synergistic activity to counteract the resistance of AML cells to FLT3-targeted therapy. In this study, we investigated the anti-leukemia activity of a MEK1 and FLT3 dual inhibitor, E6201, in AML cells resistant to FLT3 inhibition. We found that E6201 exerted profound apoptogenic effects on AML cells harboring resistance-conferring FLT3 mutations. This activity appeared to be p53 dependent, and E6201-induced cytotoxicity was retained under hypoxic culture conditions and during co-culture with mesenchymal stem cells that mimic the AML microenvironment. Furthermore, E6201 markedly reduced leukemia burden and improved the survival of mice in a human FLT3-mutated AML model. Collectively, our data provide a preclinical basis for the clinical evaluation of E6201 in AML patients harboring FLT3 mutations, including those who relapse following FLT3-targeted monotherapy.

Keywords

E6201; MEK inhibitor; FLT3 inhibitor; acute myeloid leukemia; FLT3-inhibitor resistance

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Introduction

Mutations of Fms-like tyrosine kinase 3 (*FLT3*) and *N/KRAS* genes are common in patients with acute myeloid leukemia (AML) with normal cytogenetics, and are present in 24 to 30% (1, 2) and 10 to 15% (3), respectively. *FLT3* internal tandem duplication mutations (*FLT3*-ITD) are the most common mutations, with the kinase domain (D835) mutation occurring less frequently. Similarly, *NRAS* mutation is more frequent than *KRAS* mutation in AML. These mutations in turn lead to aberrant activation of *FLT3* and/or RAS–mitogen-activated protein kinase (MAPK) pathways. Responses to single agent tyrosine kinase inhibitors of *FLT3* (e.g., quizartinib and sorafenib) (4, 5) or MAPK kinase (MEK1/2) (e.g., GSK1120212) (6) have been mostly restricted to patients with the corresponding mutations, which confirm that these mutations are valid targets. However, the treatment responses obtained with these agents have been unsustainable. Acquisition of point mutations, for which novel inhibitors are in early development, has emerged as an important mechanism of resistance to *FLT3* inhibitors. However, this mechanism does not account for all of the acquired resistance processes that have been reported (7). Indeed, the aberrant activation of parallel signaling pathways such as MAPK and AKT may also contribute to acquired resistance (8).

In our clinical trials with sorafenib, the upregulation of phospho-ERK was observed in AML cells from patients with disease relapse, suggesting that MAPK activation takes place even when *FLT3* phosphorylation remained suppressed (5). In addition, we have developed sorafenib-resistant cells by introducing clinically-relevant point mutations of *FLT3* into murine leukemia cells (e.g., Ba/F3-ITD+842 and Ba/F3-ITD+676/842) and the upregulation of phospho-ERK was also observed in these cells (9). Unfortunately, similar data are not available for patients treated with MEK inhibitors. However, preclinical data suggests that *FLT3* is upregulated when AML cells are exposed to an inhibitor of MEK signaling (10). Furthermore, concomitantly targeting *FLT3* and MEK signaling pathways has achieved encouraging synergistic anti-leukemia effects in our *in vitro* and *ex vivo* studies, suggesting a potential for preventing/overcoming relapse in patients treated with *FLT3* inhibitors like sorafenib and quizartinib (9).

E6201 is a synthetic small molecule that functions as a non-allosteric tyrosine kinase inhibitor, which inhibits both MEK1 and *FLT3* (11). E6201 shows identical affinity and residence time for the active and inactive forms of MEK1 (12), and demonstrates different pharmacologic activities than those of allosteric MEK inhibitors and exerts exclusive effects on targeting acquired MEK1-C121S mutation, which confers resistance to the allosteric MEK inhibitor selumetinib (AZD6244) in melanoma (13). E6201 also has a long occupancy time for *FLT3* (11-fold longer than that for MEK). In addition, the backbone structure of E6201 markedly differs from other allosteric *FLT3* inhibitors such as sorafenib or quizartinib (Fig S1). Thus, E6201 is an attractive clinical compound for effectively targeting leukemic cells with aberrant activation of both *FLT3* and MAPK signaling pathways, especially for those resistant to *FLT3*-inhibitors.

Here, we report that E6201 has marked cytotoxic activity against AML cells harboring *FLT3*-ITD or *NRAS* mutations. E6201 was especially effective in the killing of FLT3-inhibitor resistant cells harboring acquired point mutations of the FLT3 TKD domains. Thus, one-third of AML patients harboring FLT3 mutations may benefit from a dual MAPK/FLT3 inhibitor with potent anticancer effects, including in cells resistant to FLT3 monotherapy.

Materials and Methods

Reagents and antibodies

E6201 was provided by Eisai Inc. (Woodcliff Lake, New Jersey), sorafenib and AC220 (quizartinib) were purchased from Selleckchem (Houston, TX). The chemical structures of these agents are shown at Supplementary Figure S1. Recombinant human FLT3/FLK2 ligand (FL) was purchased from R&D (Minneapolis, MN). Interleukin-3 (IL-3) was purchased from PEPROTECH (Rocky Hill, NJ). The antibodies against human phosphorylated (p)-p44/42 MAPK (ERK1/2)(Thr202/Tyr204), phospho-AKT(Ser473), phospho-FLT3(Tyr589/591), phospho-S6K(Ser240/244), phospho-MEK1/2, AKT, S6K, Bcl-xL, and cleaved-caspase-3 were purchased from Cell Signaling Technology (Danvers, MA), against Bcl-2 from Dako (Carpinteria, CA), against phospho-STAT5 A/B from Upstate (Lake Placid, NY), against ERK2, FLT3, p53 and Mcl-1 from Santa Cruz Biotechnology (Santa Cruz, CA), against Bim and Puma from CalBiochem (San Diego, CA), and against Ki67 was purchased from Abcam (Cambridge, MA). The anti-luciferase antibody was purchased from Promega (Madison, WI).

AML cell lines and patient samples

The Ba/F3-FLT3, Ba/F3-ITD, Ba/F3-D835G and Ba/F3-D835Y cell lines (kindly provided by Dr. Donald Small in 2010, Department of Pediatric Oncology, Johns Hopkins University, Baltimore, MD) were derived from immortalized murine pro-B lymphocyte Ba/F3 cells that were transfected with lentivirus containing human FLT3 or FLT3-ITD mutations, isolated, and characterized in 2002 as described previously (14). The FLT3-inhibitor resistant cells Ba/F3-ITD+691, Ba/F3-ITD+842, and Ba/F3-ITD+676/842 cells, which harbored FLT-ITD plus F691L, Y842C, and N676D/Y842C mutations, respectively, were established in 2013 as described previously (9). The human AML cell lines THP-1, Kasumi-1, and MV4-11 were obtained from the American Type Culture Collection (Manassas, VA), OCI/AML3 cell line from Dr. M. Minden (Princess Margaret Hospital, Toronto, Ontario, Canada), MOLM13 cell line from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), MV4-11-NRAS mutated and parental cells were established in 2013 by lentivirally transfecting NRAS/G12D mutations as described previously (15). All cell lines were validated by STR DNA fingerprinting using the AmpF_STR Identifier kit according to manufacturer's instructions (Applied Biosystems cat 4322288) in September 2010. The STR profiles were compared to known ATCC fingerprints (ATCC.org), and to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (<http://bioinformatics.istge.it/clima/>) (Nucleic Acids Research 37:D925-D932 PMID: PMC2686526). The STR profiles matched known DNA fingerprints or were unique. All cells were maintained in RPMI medium supplemented with 10% fetal bovine serum, and

IL-3 dependent murine Ba/F3-FLT3 cells were maintained in the presence of 2 ng/mL of IL-3. The genetic characteristics of AML cell lines used in this study are summarized in Table 1.

Mesenchymal stem cells (MSCs), obtained from normal bone marrows (BM) following Institutional guidelines, were cultured at a density of 5,000 cells/cm² in α -MEM supplemented with 20% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. The MSCs were used for co-culture experiments after passage four.

Peripheral blood samples were obtained from patients with newly diagnosed, relapsed, or refractory AML after written informed consent had been obtained from each patient (according to the institutional guidelines of the University of Texas MD Anderson Cancer Center). The patient characteristics are presented in Supplementary Table S1. The mononuclear cells in these samples were purified by Ficoll-Hypaque (Sigma-Aldrich) density-gradient centrifugation, and the cells were cultured in RPMI 1640 culture medium supplemented with 10% fetal calf serum as described above prior to treatment.

Cell viability and apoptosis assays

Cell viability was assessed using the Trypan blue dye exclusion method, and apoptosis was determined via FACS by annexin V positivity as described previously (16). The 50% inhibitory concentration (IC₅₀) for cell growth inhibition and the 50% effective concentration (EC₅₀) for apoptosis induction were calculated using CalcuSyn software (BioSoft, Cambridge, UK). Apoptosis induction of leukemic progenitor cells (i.e., the CD34⁺ AML cells) was determined as described above by calculating specific apoptosis using the following formula: specific apoptosis (%) = 100 (drug-induced apoptosis - spontaneous apoptosis)/(100 - spontaneous apoptosis) (17).

For measuring apoptosis induction in the leukemia cells co-cultured with MSC, the cells were trypsinized and stained with CD90-PE and annexin V-Cy5 (both from BD Biosciences) and apoptosis was assessed by measuring annexin V-Cy5 positivity after excluding the CD90 (used as a MSC marker) positive cell population.

Immunoblot analyses

AML cells were treated with E6201 or sorafenib at the indicated concentrations and then collected for analysis. Semi-quantitative assessment of immunoblots was determined using the Scion Imaging system and software (Beta version 4.03; Scion, Frederick, MD) (18).

Animal study

The animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas, MD Anderson Cancer Center. NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NOG) mice (10 weeks-old female; n = 27; The Jackson Laboratory, Bar Harbor, ME) were injected intravenously with 0.5×10^6 of MOLM13-Luci-GFP cells that were lentivirally infected with firefly luciferase. Mice were treated with either E6201 (40 mg/kg, n = 14) or saline (n = 13) via tail vein at a twice-weekly schedule, which started from Day 4 to Day 21 after leukemia cell injection. Mice were noninvasively imaged in a

Xenogen-200 *in vivo* bioluminescence imaging system (Xenogen, Hopkinton, MA) after injection with a D-luciferin (4 mg/mouse) substrate. Bioluminescence imaging based on the detection of light emission was measured in photons/second and has been described in detail elsewhere (19). Three mice for each group were sacrificed on day 16 after tumor cell injection, and spleen, liver, lung, and BM samples were collected for immunohistochemistry analysis. Briefly, the collected tissues were fixed for 30 min in 10% neutral buffered Formalin solution. The tissues for immunofluorescence were dehydrated in serial concentrations of sucrose in DPBS, and frozen in 100% OCT. Sections were labeled with anti-phospho-ERK antibody (Cat # 9101, Cell Signaling Technology, Danvers, MA). The tissues were fixed in 10% neutral buffered formalin solution at 4°C overnight, then dehydrated, embedded in paraffin, and sectioned. After antigen retrieval, the slides were incubated with anti-luciferase or anti-Ki67 antibodies.

Statistical analyses

The Student *t*-test was used to analyze immunoblot, cell growth, and apoptosis data. A *P*-value 0.05 was considered statistically significant. All statistical tests were two-sided and the results are expressed as the mean of triplicate samples/experiments $\pm$ S.D./ 95% confidence intervals (error bars). The efficacy of E6201 on survival was estimated by the Kaplan–Meier method (20), and log-rank statistics was used to test for differences in survival.

Results

E6201 exerts marked anti-leukemia effects in AML cells

We investigated anti-leukemia effects of E6201 on various AML cells lines, including those with different mutational status of *FLT3* and *RAS*. E6201 profoundly inhibited the growth, and triggered apoptosis, in the *FLT3*-ITD mutant leukemic cells MOLM13 and MV4-11 (IC₅₀ 0.005 and 0.002 μ M, respectively), but showed close to 100-fold less activity against *FLT3* WT cells THP-1 and Kasumi-1 (IC₅₀ 0.67 and 0.37 μ M, respectively, Fig. 1A; Table 1). We further tested E6201 in murine leukemia cells harboring *FLT3*-ITD and/or tyrosine kinase domain (TKD) mutations. Ba/F3-ITD cells were 1,000-fold more sensitive than the parental cells Ba/F3-FLT3 (IC₅₀ 0.003 vs. 3.18 μ M). To exclude potential interference of IL3 in inhibition of Ba/F3-FLT3 cells, which are IL3-dependent, by E6201, these cells were also treated with E6201 in the absence of IL3. Ba/F3-FLT3 cells were still approximately 140-fold less sensitive than Ba/F3-ITD cells (IC₅₀ 0.42 vs. 0.003 μ M) (Fig. 1A; Table 1). Importantly, E6201 inhibited proliferation of *FLT3* TKD-mutated cells Ba/F3-D835G and Ba/F3-D835Y (IC₅₀ 0.0046 and 0.026 μ M, respectively) (Fig. 1A; Table 1). These TKD mutations usually show more resistance to *FLT3*-targeting therapy in clinic. Specifically, compared to the clinically-potent *FLT3* inhibitor quizartinib (21), E6201 demonstrated more potent anti-proliferative effects in leukemic cells harboring these TKD mutations (Supplementary Table S2). In addition, E6201 also demonstrated strong anti-proliferative effects in *FLT3*-inhibitor resistant cells, which carry acquired point mutations in the kinase domains in addition to *FLT3*-ITD mutations, such as Ba/F3-ITD+691, Ba/F3-ITD+842 and Ba/F3-ITD+676/842 (IC₅₀ 0.1, 0.0089, and 0.0056 μ M, respectively), and induced profound apoptosis in these cells as well (Table 1, Fig. 1A). We compared the sensitivities of these

resistant cells and their parental Ba/F3-ITD cells to E6201 and sorafenib. E6201 exerted marked pro-apoptotic effects relative to that induced by sorafenib (i.e., ~ 124- to 327-fold higher activity, Table 2) in the resistant cell lines. Immunoblot analysis demonstrated that E6201 significantly suppressed phospho-FLT3, phospho-ERK, and phospho-STAT5, which was accompanied by the downregulation of Mcl-1, Bcl-xL and activation of caspase-3 (Fig. 1B and C). To further evaluate if E6201 had better effectiveness in targeting MAPK than other FLT3 inhibitors which might be more dependent on their suppression of FLT3 signaling pathway and then its cascade downstream MAPK, ITD mutated cells Ba/F3-ITD, FLT3-inhibitor resistant cells Ba/F3-ITD+691 and Ba/F3-ITD+676/842 were treated at their IC₅₀ concentrations to individual cell lines. Immunoblot analysis showed that E6201 had more effective inhibition of phospho-ERK than sorafenib or quizartinib (AC220) in the resistant cell lines although it showed same suppression level of phospho-FLT3 to each drugs at their IC₅₀ concentrations (Fig. 1D). The results imply that E6201 has direct suppressing effect of phospho-ERK in addition to targeting FLT3 signaling pathway, which is more effective in FLT3-inhibitor resistant cells and may benefits for overcoming the resistance in these cells.

Activation of MEK/ERK signaling pathway by NRAS mutation sensitizes E6201-induced pro-apoptotic effects in AML cells

Because E6201 demonstrated more effectiveness of MAPK inhibition compared with other FLT3 inhibitors, we next investigated its effects against AML cells that harbor *RAS* mutations. Interestingly, the *NRAS* mutant OCI/AML3 cells, which usually show resistance to many other FLT3 inhibitors in our hands (22), also demonstrated higher sensitivity to E6201 (IC₅₀ 0.037 μ M, Table 1), suggesting that high ERK activation might be responsible for sensitivity to E6201 treatment (15). Furthermore, E6201 triggered higher apoptosis induction in MV4-11-*NRAS*-mut cells than in their parental controls (Fig. 2A). The MV4-11-*NRAS*-mut cells have higher basal level of phospho-ERK, lower basal levels of phospho-FLT3 and -STAT5 relative to the *NRAS* WT cells. A 6-h E6201 treatment resulted in more suppression of phospho-ERK, but not phospho-FLT3 or phospho-STAT5 (Fig. 2B). Furthermore, a 24-h treatment triggered profound upregulation of pro-apoptotic protein Bim and caspase-3 cleavage (Fig. 2C), suggesting an enhanced pro-apoptotic effect in *NRAS* mutant AML cells, also implying that an inhibition of MAPK signaling might be indispensable in these cells.

E6201 exerts pro-apoptotic effects in AML patient samples with FLT3-ITD mutations—We next tested cytotoxicity of E6201 in eight AML blast samples with different *FLT3* and *NRAS* mutational profiles. The results shown in Figure 3A illustrated that E6201 exerted marked cytotoxic effects in all of the five patient samples tested with *FLT3*-ITD mutations, including in one patient sample with dual *FLT3*-ITD and *NRAS* mutations. Three of these samples were obtained from patients with relapsed/refractory disease (Supplementary Table S1). E6201 induced modest apoptosis in AML patient samples with *FLT3* D835 mutation, and had only a minor apoptotic effect on WT *FLT3* cells (Fig. 3A). Of note, E6201 also did not show pro-apoptotic effects on normal bone marrow (Supplementary Fig. S2). Immunoblot analysis of the AML patient samples after *ex vivo* treatment with E6201 for 24 h illustrated a strong suppression of FLT3, AKT, and ERK

phosphorylation, increased levels of the pro-apoptotic protein Bim, and the down regulation of the anti-apoptotic protein Mcl-1 in *FLT3*-ITD mutant cells compared to their *FLT3* WT counterparts (Fig. 3B).

E6201 abrogates protection mediated by FL, bone marrow stromal cells, and hypoxia in *FLT3* mutated AML cells—FL induction has been reported as a cellular response of AML cells to chemotherapy and it is associated with resistance to *FLT3*-targeted therapy (23). We tested the effects of E6201 on the *FLT3*-ITD mutated/*FLT3*-inhibitor resistant cells. We observed that exogenous FL (25 ng/mL) reduced the sorafenib-induced apoptotic effects, especially in the *FLT3*-inhibitor resistant Ba/F3-ITD+676/842 cells ($P < 0.05$). However, E6201 abrogated the protective effects of exogenous FL and demonstrated similar pattern of apoptosis induction as those without FL treatment (Fig. 4A). Of note, the Ba/F3-ITD+676/842 cells demonstrated a higher basal level of phospho-ERK in the presence of FL, suggesting that E6201 sensitivity may be due to the MEK inhibitory activity rather than *FLT3* inhibition. Immunoblot analysis showed that E6201 was more effective in suppressing phospho-ERK and downregulating anti-apoptotic protein Mcl-1 in the presence of FL compared to sorafenib treatment alone in Ba/F3-ITD+676/842 cells (Fig. 4B). These results suggest that E6201 can effectively abrogate the putative protective effects of FL, as a potential resistance mechanism, in AML cells with *FLT3* mutations.

Components of the BM microenvironment, including MSCs and hypoxia, can also provide protection for leukemia cells during chemotherapy (24). Therefore, we investigated the impact of co-culturing the AML cells with MSCs under hypoxic condition on E6201-triggered apoptosis induction. Ba/F3-ITD+676/842 cells were exposed to E6201 in hypoxic or normoxic environments with or without MSCs for 24 h, and phospho-*FLT3*, phospho-ERK, Bim, and cleaved caspase-3 levels were measured by immunoblotting. Results showed that hypoxia increased phospho-*FLT3* levels (1.7-fold) and co-culture with MSCs under these conditions further enhanced the phospho-ERK levels by 2.5-fold (Fig. 4C). E6201 treatment profoundly suppressed phosphorylation levels of *FLT3* and ERK in hypoxia, and the presence of MSCs (2.2-fold and 2.3-fold in phospho-*FLT3* and phospho-ERK levels, respectively), and markedly increased levels of pro-apoptotic Bim and cleaved caspase-3 (Fig. 4C). Further, E6201, in a dose-dependent manner, significantly increased the pro-apoptotic effects of hypoxia compared to normoxia in the presence of MSCs ($P < 0.01$, Fig. 4D). These data suggest that E6201 can overcome the protection of the BM microenvironment during leukemia therapy.

E6201 suppresses AML *in vivo*—We further assessed the *in vivo* efficacy of E6201 in a murine leukemia model. NOG mice bearing xenografts of MOLM13-Luc-GFP cells were examined. E6201 treatment (40 mg/kg following a twice-weekly schedule via tail vein injection) for three weeks significantly reduced leukemia burden compared to the vehicle group (i.e., the mean luminescence at d 14: 2.7×10^6 photons/sec vs. 5.6×10^6 photons/sec in the E6201-treated group vs. vehicle-treated group, Fig. 5A and B). The median survival was 25 days for the E6201-treated group vs. 18 days in the vehicle-treated group ($p < 0.0001$, Fig. 5C). In addition, no signs of weight loss or related toxicities were observed in the treatment cohort (Supplementary Fig. S3). Furthermore, immunohistochemical analysis

demonstrated that E6201 treatment reduced leukemia cell infiltration and proliferation in organs including bone marrow, lung, liver and spleen (Fig. 5D, top and middle panels). Meanwhile, E6201 treatment demonstrated a profound suppression of phosphorylated ERK in the engrafted leukemic cells found in lung and liver analyzed by immunofluorescence staining with anti-phospho-ERK antibody (Fig. 5D, bottom panel).

Discussion

Several mechanisms have been implicated in the acquired resistance to FLT3-targeted therapy. One of them involves acquired point mutations in the TKDs, which has been reported by our group and others (9, 25, 26). In addition, we previously reported that high levels of phosphorylated ERK persisted in leukemia blasts after therapy with sorafenib despite continued suppression of phospho-FLT3 (5), suggesting that high MAPK activation might constitute another mechanism of resistance. High ERK activation was also observed in the FLT3-inhibitor resistant cell lines (9), and this was further increased by co-culture of these cells with MSCs in hypoxic conditions (2.5-fold higher, Fig. 4C). E6201 exerted profound pro-apoptotic effects not only in the *FLT3*-ITD mutant cells, but also in the FLT3-inhibitor resistant cells including the Ba/F3-ITD+691 cells (Fig. 1A, Table 2). The latter cells harbor a ‘gate keeper’ mutation (F691L) in the TKD that is responsible for sorafenib and quizartinib resistance (27). Importantly, E6201 more effectively suppressed ERK phosphorylation compared with the other FLT3 inhibitors sorafenib and quizartinib in FLT3-inhibitor resistant Ba/F3-ITD+691 and Ba/F3-ITD+676/842 cells, suggesting a direct suppressive effectiveness on MAPK signaling in addition to FLT3 signaling (Fig 1A, D, supplementary Fig. S4). In fact, concomitantly targeting FLT3 and MEK with FLT3 inhibitor sorafenib and MEK inhibitor CI-1040 exerted synergistic pro-apoptotic effects in the FLT3-inhibitor resistant AML cells (9). Thus, using the dual FLT3/MEK inhibitor E6201 may provide an optimal effect on apoptotic synergism and reduce potential side effects caused by using multiple drugs combinations.

E6201 was also active against *NRAS* mutant cells. In our system, the introduction of mutant *NRAS* raised the basal level of phospho-ERK, which might be the dominant survival signaling pathway in these cells. Specifically, reduction of the basal phospho-FLT3 level was observed in MV4-11-*NRAS*-mutated cells compared to their parental WT cells (Fig 2B). Therefore, the direct effectiveness of targeting MAPK with E6201 may contribute to the sensitivity of AML cells with mutant *NRAS*. However, we noticed that THP-1 and Kasumi-1 cells, which harbor *NRAS* or *KRAS* mutations, demonstrated less sensitivity to E6201 compared to OCI/AML3 cells that harbor *NRAS* mutations. THP-1 and Kasumi-1 also have mutant *TP53*, but OCI/AML3 harbors wild type *TP53* [http://p53.free.fr/Database/Cancer_cell_lines/cell_lines_1.0.pdf]. To clarify the impact of p53 status on E6201 sensitivity, we compared parental AML cell lines to their p53 knockdowns and demonstrated that WT p53 protein was critical for sensitizing these cells to E6201-induced apoptosis (Supplementary Fig. S5).

The interactions between the tumor suppressor p53 and MAPK signaling are still unclear. Several studies have postulated that activation of MAPK signaling can trigger increased p53 mRNA levels via activation of its upstream functional protein C/EBP- β (28-30) or directly

through phosphorylation of p53 at Thr55 (31). Our *NRAS* mutant cells also showed increased p53 levels compared to *NRAS* wild type cells (Fig. 2B). MAPK signaling is also involved in the nuclear localization and transportation of p53 (32). Conversely, p53 as an upstream protein also enables the upregulation of MAPK signaling (33). MAPK signaling is also involved in the nuclear localization and transportation of p53 (34). Our data strongly support the notion that the aberrant activation of phospho-ERK in the *NRAS* mutated cells is accompanied by higher levels of p53 protein (Fig. 2B), and that high p53 levels maintain a potentially higher basal level of phospho-ERK in *TP53* WT cells (Fig. 2B).

The tumor suppressor p53 mediates cell apoptosis as a nuclear transcription factor (35) or by transcription-independent mechanisms such as physical interaction between p53 and Bcl-2 family proteins (36, 37). p53 has a high binding affinity to anti-apoptotic Mcl-1 and a weak binding affinity to Bcl-xL via different p53 transcription activation domains (38). p53 reportedly binds Mcl-1 to liberate pro-apoptotic Bim or Bak to trigger mitochondria-mediated apoptosis (39). In addition, the activation of MAPK signaling has been reported to promote expression of anti-apoptotic Mcl-1 and to stabilize the protein (40, 41), which has a very short half-life (42). Thus, the downregulation of p53 protein, or phosphorylation level of p53Thr55, via the suppression of MEK signaling with E6201 may limit the binding capacity of Mcl-1 and lead to quick degradation of Mcl-1 as shown in *NRAS* mutated *TP53* WT cells [Fig. 2B and C]. Therefore, the exclusivity of E6201 in targeting MAPK provides a mechanistic rationale for apoptosis induction via suppression of phospho-ERK, the downregulation of Mcl-1, and the upregulation of Bim as shown in Figure 2B and C. Given the high frequency of *NRAS* mutations (10 to 30%) and of WT *TP53* (90%) in myeloid malignancies, this would suggest that E6201 could be highly active in this large subset of myeloid malignancies.

FL is another upstream activator of the FLT3-MAPK signaling axis (43). The presence of exogenous FL increases basal levels of ERK phosphorylation as shown in Ba/F3-ITD +676/842 cells (Fig. 4B). Concomitantly targeting FLT3 and MEK with the FLT3 inhibitor crenolenib and the MEK inhibitor CI1040 triggered marked apoptosis in the FLT3-inhibitor resistant cells (9). Validation of the concept of combined targeting of FLT3 and MEK is further substantiated by this study of E6201. Mechanistically, targeting MEK/FLT3 induces leukemia cell death by modulating the MEK-Mcl-1-Bim signaling axis.

FL-mediated proliferation, differentiation, and survival of hematopoietic stem and progenitor cells are dependent on co-operation with other growth factors such as IL3 etc. (44, 45). Importantly, E6201 demonstrated no effect on IL3-dependent pro-B Ba/F3-FLT3 cells in the presence of FL which reflects the physiological situation of the bone marrow (data not shown). Taken together, E6201 can overcome FL-mediated resistance in FLT3 mutant AML but shows limited effect on FLT3 wild type cells, suggesting that its toxicity against normal hematopoietic stem and progenitor cells is likely to be minimal.

The BM microenvironment plays a critical role in the chemoresistance of AML (46). As it has been shown hypoxia strikingly increases phosphorylated FLT3 level and the presence of MSCs and exclusively upregulates ERK activation and further upregulated Mcl-1 (47, 48), implying upregulation of MEK/ERK and FLT3 signaling pathways are key factors in the

microenvironment-induced AML cell resistance to chemotherapy. Therefore, concomitantly targeting MEK and FLT3 with E6201 provides the possibility of significantly enhancing the efficacy of FLT3-targeted therapy by overcoming BM-mediated activation of MAPK, which may provide new therapeutic approach for treating relapsed and refractory AML patients. A Phase 1/2a clinical trial for evaluating anti-leukemia efficacy of E6201 on targeting relapsed/refractory FLT3 mutated AML is ongoing currently in MD Anderson Cancer Center.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

E6201 triggers anti-leukemia effects in AML cells with *FLT3* mutations. (A) Human and murine AML cell lines cells were treated with E6201 at the indicated concentrations for 72 h. Apoptosis induction and cell-growth inhibition were assessed as the percentage of annexin V-positive cells by flow cytometry and by counting the numbers of viable cells using the Trypan blue dye exclusion method, respectively. Growth inhibition was expressed as percentage relative to that in the control group. Data are the mean of 3 independent determinations. Error bars correspond to 95% confidence intervals. (B) The levels of phosphorylated and total FLT3, ERK, STAT5 and S6K proteins were determined by immunoblotting after treatment with E6201 for 1 h. (C) The levels of apoptosis-related

proteins Mcl-1, Bcl-xL, Bim-EL (extra-long), -L (long) and cleaved caspase-3 (cleaved-C3) were determined by immunoblotting after treatment with E6201 for 24 h. (D) Leukemic cells were treated with E6201, sorafenib (Sora) or AC220 at IC_{50} concentrations of each drug to individual cell lines for 18 h, and the modulation of phospho-FLT3 and -ERK levels was evaluated by immunoblotting. The ratios are semi-quantitative analyses indicating the expression levels of phosphorylated proteins compared to their total proteins. The expression levels were calculated relative to that in control lane of each cell lines, which was defined as 1. GAPDH or tubulin was used as loading control.

Figure 2.

The mutational status of *NRAS* affects AML sensitivity to E6201-induced cell death. (A) *NRAS*-WT and *NRAS*-mutant MV4-11 cells were treated with E6201 at the indicated concentrations (μM) for 48 h, and examined for apoptosis induction (annexin V positivity) as described in Figure 1. Inset: basal phosphorylation level of phospho-MEK in WT and *NRAS*-mutated MV4-11 cells. * $P < 0.05$. *NRAS*-WT and *NRAS*-mutant MV4-11 cells were treated with E6201 for (B) 6 h or (C) 24 h, and the levels of correlated phosphorylated proteins or apoptosis-related proteins were measured by immunoblotting. The ratios of semi-quantitative analyses indicated the expression levels of phosphorylated proteins to their total proteins or housekeeping proteins GAPDH (or Actin). GAPDH or actin was used as loading control. Cleaved-C3; cleaved caspase-3.

Figure 3. E6201 is apoptogenic in AML blasts with *FLT3*-ITD mutations. (A) Mononuclear cells obtained from the peripheral blood of AML patients with differing *FLT3* mutational status were exposed to E6201 at the indicated concentrations for 48 h. Apoptosis induction (annexin V positivity) was determined as described in Figure 1. (B) Blasts of AML patients harboring WT *FLT3* or *FLT3*-ITD were treated with the indicated concentrations of E6201 for 24 h, and the indicated protein expression was determined by immunoblotting. Semi-quantitative analyses were described as above. *Percentage of specific apoptosis induction.

Figure 4.

E6201 diminishes the protection mediated by FL, MSCs, and hypoxia. (A) Ba/F3-ITD cells and Ba/F3-ITD+676/842 cells were treated with E6201 or sorafenib for 48 h in the presence/absence of FL, and apoptosis induction (annexin V positivity) was determined as described in Figure 1. NS = not statistically significant; * $P < 0.05$. (B) The indicated protein expression was determined by immunoblotting after 24 h of treatment with the indicated concentrations of E6201 or sorafenib in the presence/absence of FL. (C) Ba/F3-ITD+676/842 cells were treated with DMSO or E6201 for 24 h in either normoxic or hypoxic environments in the presence/absence of a MSCs feeder layer. The levels of phospho-FLT3, -ERK, Bim and cleaved-caspase-3 were determined using immunoblotting. The expression levels (ratios of indicated proteins to housekeeping protein Tubulin) were calculated relative to that in the first lane which was defined as 1. Cleaved-C3; cleaved caspase-3. (D)

Ba/F3/ITD+676/842 cells were treated with E6201 for 48 h in the presence/absence of MSCs and apoptosis induction as described above. NS = not statistically significant; $**P < 0.01$.

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Figure 5.

E6201 exerts anti-leukemia activity *in vivo*. (A) Serial bioluminescence images of representative mice at 4 and 14 d after injection with MOLM13-Luci-GFP cells. (B) The mean peak light-emission from each group displayed in terms of photons/sec. (C) Kaplan-Meier estimates of overall survival in mice treated with E6201 (n = 11) or vehicle (n = 10) via intravenous injection from Day 4 to Day 21 following a schedule of twice per week. The log-rank (Mantel-Cox) test was made to compare survival curves between the vehicle-treated group and the E6201 (40mg/kg) treatment ($\chi^2 = 16.88$, degrees of freedom = 1, $P < .0001$). The median survival were assessed at 18 days and 25 days ($p < 0.0001$), respectively, in the vehicle-treated group and the E6201 treatment group. The arrowhead indicates leukemic cells injection. The dash arrow bar indicates duration of E6201 administration. (D) Histological analysis by immunohistochemical (IHC) staining with anti-luciferase antibody for detecting infiltrated leukemic cells (top panel, positive brown stain indicates leukemia cells), and with anti-Ki67 antibody for detecting proliferating cells (middle panel, positive brown cells indicates proliferating cells) on slides of bone marrow, lung, liver, and spleen, which were collected at Day 16 mice after leukemic cells injection. Bottom panel was stained with immunofluorescence (IF) after incubating with anti-phospho-ERK (p-ERK) antibody (Cat # 9101, Cell Signaling Technology, Danvers, MA) on sections of lung and liver collected on Day 16 mice after leukemic cells injection.

Table 1

The half maximal inhibitory concentrations (IC₅₀s) of E6201 in leukemia cell lines.

	Cell Line	Type	<i>FLT3</i>	<i>RAS</i>	IC ₅₀ (μm)	Lower 95%	Upper 95%
Murine cells	Ba/F3-FLT3(+IL3)	Murine pro-B	WT	WT	3.18	1.96	5.16
	Ba/F3-FLT3 [*]	Murine pro-B	WT	WT	0.42	0.19	0.97
	Ba/F3-ITD	Murine pro-B	ITD mut	WT	0.0031	0.00244	0.00415
	Ba/F3-ITD+691	Murine pro-B	ITD+F691L mut	WT	0.1	0.05	0.199
	Ba/F3-ITD+842	Murine pro-B	ITD+Y842C mut	WT	0.0089	0.0076	0.0105
	Ba/F3-ITD+676/842	Murine pro-B	ITD+N676D+Y842C mut	WT	0.0056	0.00017	0.18
	Ba/F3-D835G	Murine pro-B	D835G mut	WT	0.0046	0.0018	0.0119
	Ba/F3-D835Y	Murine pro-B	D835Y mut	WT	0.026	0.0035	0.1866
Human Cells	MOLM13	AML	ITD mut	WT	0.0049	0.00303	0.00712
	MV4-11	Biphenotypic Leukemia	ITD mut	WT	0.0019	1.26E-05	0.25209
	THP-1	Myeloid Leukemia	WT	<i>NRAS</i> /12 mut	0.67	0.11	4.17
	Kasumi-1	Myeloid Leukemia	WT	<i>KRAS</i> /12 mut	0.37	0.11	1.22
	OCI-AML3	Myeloid Leukemia	WT	<i>NRAS</i> /61 mut	0.037	0.0091	0.182

* Ba/F3-FLT3 cells were treated with E6201 in the absence of interleukin 3 (IL3) growth factor; mut = mutations; WT = wild type; N/A = not applicable

Table 2Comparison of EC₅₀s in murine ITD mutant and TKDs mutant cell lines

Cell Line	Sorafenib (μmol/mL)		E6201 (μmol/mL)	
	EC ₅₀ (95% Lower/Upper)	Fold to Baf3-ITD *	EC ₅₀ (95% Lower/Upper)	Fold to Baf3-ITD *
Ba/F3-ITD	0.006 (0.004/0.009)	1	0.084 (0.022/0.316)	1
Ba/F3-ITD+691	5.08 (4.33/5.97)	846	0.57 (0.37/0.86)	6.79
Ba/F3-ITD+842	2.60 (1.91/4.25)	433	0.123 (0.049/0.305)	1.46
Ba/F3-ITD+676/842	6.60 (5.50/7.61)	1100	0.282 (0.092/0.856)	3.36

* Comparison of EC₅₀s of resistant cell lines to their parental Baf3-ITD cells in sorafenib and E6201 treatment, respectively.