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Breakdown of the FLT3-ITD/STAT5 Axis and Synergistic Apoptosis Induction by the Histone Deacetylase Inhibitor Panobinostat and FLT3-Specific Inhibitors

Kristin Pietschmann¹, Hella Anna Bolck¹, Marc Buchwald¹, Steffi Spielberg¹, Harald Polzer³, Karsten Spiekermann³, Gesine Bug⁴, Thorsten Heinzel¹, Frank-Dietmar Böhmer², and Oliver H. Krämer¹

Abstract

Activating mutations of the class III receptor tyrosine kinase FLT3 are the most frequent molecular aberration in acute myeloid leukemia (AML). Mutant FLT3 accelerates proliferation, suppresses apoptosis, and correlates with poor prognosis. Therefore, it is a promising therapeutic target. Here, we show that RNA interference against FLT3 with an internal tandem duplication (FLT3-ITD) potentiates the efficacy of the histone deacetylase inhibitor (HDACi) panobinostat (LBH589) against AML cells expressing FLT3-ITD. Similar to RNA interference, tyrosine kinase inhibitors (TKI; AC220/cpd.102/PKC412) in combination with LBH589 exhibit superior activity against AML cells. Median dose–effect analyses of drug-induced apoptosis rates of AML cells (MV4-11 and MOLM-13) revealed combination index (CI) values indicating strong synergism. AC220, the most potent and FLT3-specific TKI, shows highest synergism with LBH589 in the low nanomolar range. A 4-hour exposure to LBH589 + AC220 already generates more than 50% apoptosis after 24 hours. Different cell lines lacking FLT3-ITD as well as normal peripheral blood mononuclear cells are not significantly affected by LBH589 + TKI, showing the specificity of this treatment regimen. Immunoblot analyses show that LBH589 + TKI induce apoptosis via degradation of FLT3-ITD and its prosurvival target STAT5. Previously, we showed the LBH589-induced proteasomal degradation of FLT3-ITD. Here, we show that activated caspase-3 also contributes to the degradation of FLT3-ITD and that STAT5 is a direct target of this protease. Our data strongly emphasize HDACi/TKI drug combinations as promising modality for the treatment of FLT3-ITD-positive AMLs. *Mol Cancer Ther*; 11(11); 2373–83. ©2012 AACR.

Introduction

Class III receptor tyrosine kinases (RTK) critically regulate the development and maturation of hematopoietic progenitor cells (1, 2). Activating mutations of the RTK FMS-like tyrosine kinase 3 (FLT3) occur in 30% to 40% of adult patients with acute myeloid leukemia (AML). Most activating FLT3 mutations in AML are internal tandem duplications (ITD) in the juxtamembrane domain of the

receptor (1, 2). FLT3-ITD mutations are independent prognostic markers for poor clinical outcome of AMLs (3–5). Mutant FLT3 critically regulates leukemic transformation by accelerating proliferation and suppressing apoptosis (1, 2), and a recent study revealed that FLT3-ITD is a driver mutation in human AMLs (6). These findings highlight the therapeutic relevance of FLT3-ITD.

A transcription factor critically contributing to transforming effects of FLT3-ITD is its target STAT5 (7). Therefore, aberrantly activated FLT3 and its downstream signaling pathways represent promising molecular targets for AML therapy. Small-molecule tyrosine kinase inhibitors (TKI) with different degrees of specificity for FLT3 have been developed. Several of these compounds have already shown anti-leukemic activity in clinical trials (8). For example, the broad-spectrum kinase inhibitor PKC412 is active against cells expressing mutant FLT3 (9, 10). Another TKI blocking FLT3 is the bis(1*H*-2-indolyl)-1-methanone compound (cpd.) 102. This agent has higher selectivity for FLT3 but a lower potency than PKC412 and strongly induces apoptosis of primary leukemic blasts and FLT3-ITD-positive murine myeloid cells (11). The most potent and specific FLT3 TKI to date is AC220 with 10-fold higher potency and selectivity than PKC412 (12–14). This second-generation TKI has recently been

Authors' Affiliations: ¹Institute of Biochemistry and Biophysics, Center for Molecular Biomedicine, Friedrich Schiller University, Jena; ²Institute of Molecular Cell Biology, Center for Molecular Biomedicine, Jena University Hospital, Jena; ³Helmholtz Zentrum München/Klinikum Großhadern, KKG Leukämie, München; and ⁴Department of Medicine, Hematology/Oncology, Goethe University Frankfurt, Frankfurt, Germany

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K. Pietschmann and H.A. Bolck contributed equally to this work.

Current address for H.A. Bolck: Institute of Molecular Cancer Research, University of Zurich, Zurich 8057, Switzerland.

Corresponding Author: Oliver H. Krämer, Institute of Biochemistry and Biophysics, Center for Molecular Biomedicine, Friedrich-Schiller University, Hans-Knöll-Str. 2, Jena 07745, Germany. Phone: 49-3641-949362; Fax: 49-3641-949352; E-mail: Oliver.Kraemer@uni-jena.de

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investigated in a phase I trial involving patients with relapsed or refractory AML and showed promising results (15). However, complete remissions were rarely achieved with FLT3 TKI monotherapy. This treatment modality frequently resulted in the development of inhibitor-resistant FLT3 mutations (6, 9, 16). Therefore, combinatorial approaches with FLT3 inhibitors and other chemotherapeutic agents may be a valuable strategy for the treatment of AML (17–20).

Histone deacetylase inhibitors (HDACi) are promising epigenetic cancer drugs, which affect gene expression and signaling by inducing acetylation of histones and non-histone proteins. Thereby, they counteract the deregulation of HDAC enzymes often observed in cancer. Moreover, HDACi alter the functions and turnover of oncoproteins (21–23). Panobinostat (LBH589) is a novel hydroxamic acid–based HDACi that inhibits zinc-dependent HDACs (classes I/–II/–IV; ref. 24). Of note, LBH589 is one of the most potent HDACi tested in clinical trials. While LBH589 shows favorable activity against various hematologic malignancies (25, 26), it became apparent that HDACi produce best results when they are applied in combination with other drugs (20). Bali and colleagues showed a superior activity of the HDACi LAQ824/PKC412 combinations in FLT3-ITD–positive cells and found that STAT5 DNA-binding activity is decreased by this treatment (18). Nishioka and colleagues noted that the HDACi MS-275 is active against AML cell lines expressing FLT3-ITD (27). In a previous study, we showed that LBH589 upregulates the ubiquitin conjugase UBCH8 mediating poly-ubiquitinylation of FLT3-ITD. Consequently, FLT3-ITD undergoes proteasomal degradation in AML cells (20). FLT3-ITD degradation was also reported to occur via HDACi-induced inhibition of the chaperone HSP90 (18, 27). Combinations of LBH589 plus TKI, in particular AC220, have not been tested to date.

On the basis of these observations, we aimed at the identification of a multi-hit strategy against mutant FLT3, which may be superior to single treatment. We considered that a more rapid and efficient inactivation of oncogenic FLT3 by inhibition of kinase activity, combined with the induction of its degradation by LBH589, may be an efficient approach to eliminate FLT3-ITD–positive AML cells. In addition, we assessed the impact of this treatment strategy on the critical prosurvival factor STAT5. Our findings reveal a strong synergistic induction of apoptosis by LBH589 in combination with all tested TKI. Compared with single-inhibitor treatment, we also found pronounced protein degradation of FLT3-ITD and STAT5 upon combinatorial treatment. Complementary to previous findings (18, 20, 27), caspases contribute to the decay of these prosurvival factors.

Materials and Methods

Drugs and chemicals

LBH589 was a gift from Novartis; compound 102 (cpd.102) is described in the work of Mahboobi and

colleagues (28); AC220 was purchased from SYNthesis med chem; PKC412 was from Enzo Lifesciences; the pan-caspase inhibitor Z-VAD-FMK was from Bachem; Amaxa Nucleofector Kit from Lonza; and polyethylenimine and propidium iodide (PI) were from Sigma-Aldrich.

Cell lines

HEK293T cells were maintained in Dulbeccos' Modified Eagle's Medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 2% L-glutamine. MV4-11, MOLM-13, RS4-11, K562, and U937 cell lines as well as peripheral blood mononuclear cells (PBMC) were grown in Roswell Park Memorial Institute medium containing same additives. All cell lines were cultured at 37°C in a 5% CO₂ atmosphere. Cell lines were not further authenticated, but all cell batches were cultivated less than 6 months from the original cell stocks.

Plasmids, siRNAs, and transfection assays

The following plasmid encoding cleaved caspase-3 is described in the work of Krämer and colleagues (29). To lower FLT3-ITD protein levels by RNA interference, MV4-11 cells were transfected with Amaxa Nucleofector Kit using program A-30, Solution V, and ON-TARGETplus SMARTpool (Dharmacon, Thermo Fisher Scientific; catalogue no. L-003137-00-0005).

Flow cytometry for apoptosis analysis and calculation of synergism

PI-fluorescence-activated cell-sorting (FACS) analyses for detection of apoptotic cell death were conducted as described (30). Annexin V staining was conducted as stated in the Supplementary Material.

Drug synergism for LBH589 and TKI (cpd.102, AC220, PKC412) was determined using sub-G₁ fractions and the median dose–effect analysis according to Chou and Talalay using CalcuSyn software (Biosoft). Synergistic interactions were defined as those having a combination index (CI) < 0.9 (31).

Caspase cleavage assay *in vitro*

Active caspase-3 was expressed in *Escherichia coli* (29). Whole-cell lysates from HEK293T overexpressing STAT5 were incubated with *E. coli* lysates containing caspase-3 in cleavage buffer [100 mmol/L Tris, pH 8.0, 10% sucrose, 150 mmol/L NaCl, 0.1% CHAPS, 10 mmol/L dithiothreitol (DTT)] and incubated at 30°C for 0 to 60 minutes. Reactions were stopped by addition of SDS-PAGE sample buffer.

Immunoblot, antibodies, and densitometric analyses

Immunoblot experiments were carried out as described (20). Antibodies were purchased from Santa Cruz Biotechnology (caspase-3, #sc-7272; FLT3, #sc-480; GFP, #sc-9996), Sigma-Aldrich (tubulin, #T5168; β -actin, #A5441), New England Biolabs [cleaved caspase-3, #9664; caspase-6, #9762; pSTAT5(a+b), #9351], Covance (HA-Tag HA.11,

#MMS-101P), BD Biosciences [PARP, #556362; STAT5 (a+b) #610191], and Biozol (Vinculin, #BLZ03106). Densitometric analyses were conducted as described in the work of Buchwald and colleagues (20).

Quantitative real-time PCR

Cellular mRNA was isolated and cDNA was synthesized as explained previously (29). Quantitative PCR (qPCR) was carried out using Absolute QPCR SYBR Green Fluorescein Mix (Thermo Scientific). Data obtained were analyzed with the ΔC_q quantification model (31) using 2 reference genes (*HMBS* and *RPL13A*). These were verified with the geNorm program (32, 33). Primer sequences for qPCR: FLT3 fwd 5'-TTTACCCCACTTTCCA-ATCACAT and rev 5'-CGAGTCCGGGTGTATCTGAAC; STAT5a fwd 5'-TGTGCCCCCAGGCTCCCTATA and rev 5'-GGCGGGAGTCAAGACTGTCCATT; RPL13A fwd 5'-CCTGGAGGAGAAGAGGAAAGAGA-3' and rev 5'-TTGAGGACCTCTGTATTGTCAA-3'; HMBS fwd 5'-GGCAATGCGGCTGCAA-3 and rev 5'-GGGTACCCACGCGAATCAC-3'

Results

FLT3-ITD knockdown sensitizes AML cells for HDACi-induced apoptosis

FLT3-ITD is an important survival factor for leukemic cells. We and others have previously shown that treatment of AML cells with the HDACi LBH589 induces degradation of this mutated receptor (17, 20). We confirmed the activity of LBH589 in our experimental setting by immunoblotting for acetylated histones H3 and H4 (Supplementary Fig. S1).

We considered that additional specific targeting of FLT3-ITD may further increase the pro-apoptotic actions of LBH589. To assess this possibility, we lowered endogenous FLT3-ITD levels in MV4-11 cells by transfecting siRNAs targeting *FLT3* mRNA before LBH589 treatment. Immunoblots for FLT3-ITD verified an efficient siRNA-mediated downregulation of FLT3-ITD protein expression (Fig. 1A). The combinatorial application of FLT3 knockdown and LBH589 treatment resulted in a very pronounced induction of apoptosis showed by cleavage and activation of caspase-3. We further determined apoptosis induction of MV4-11 cells by FACS analysis (measuring apoptotic sub- G_1 fractions with DNA contents below 2N). Treatment with low doses of LBH589 only marginally affected control siRNA-transfected cells. Remarkably, MV4-11 cells with reduced FLT3-ITD expression were more strongly affected by LBH589 than control siRNA-transfected cells (Fig. 1B). Similar results were gained using an independent siRNA targeting *FLT3* (Supplementary Fig. S2).

We additionally found that the combination of FLT3-ITD knockdown and LBH589 treatment decreased the protein levels of the transcription factor STAT5 (Fig. 1A). This anti-apoptotic signal transducer is an essential survival factor for leukemic cells (2, 7, 34), and its reduction in treated MV4-11 cells correlates with the activation

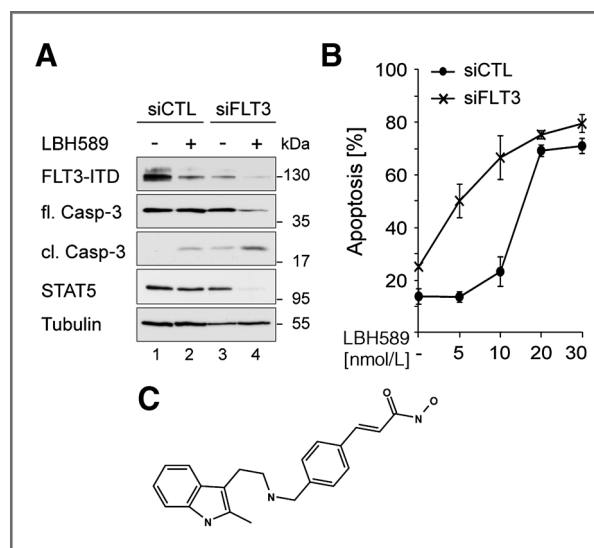


Figure 1. Depletion of FLT3-ITD enhances LBH589-induced apoptosis and STAT5 degradation in AML cells. **A**, MV4-11 cells were transfected with siRNAs directed against *FLT3* mRNA or unspecific siRNAs as a control (siCTL). After 16 hours, cells were treated with 30 nmol/L LBH589 (+) or dimethyl sulfoxide (–) for another 16 hours. Expression of FLT3-ITD, STAT5, and caspase (Casp)-3 (cl, cleaved; fl, full-length) was analyzed by immunoblotting. The antibody binding STAT5 recognizes STAT5a as well as STAT5b. Tubulin served as loading control. **B**, MV4-11 cells were transfected with siRNAs directed against *FLT3* mRNA or unspecific siRNAs as a control (siCTL). After 4 hours, cells were treated with 5 to 30 nmol/L LBH589 or DMSO (–) for another 24 hours. Apoptosis rates were determined by PI-FACS analysis. Diagram shows sub- G_1 fractions (apoptotic cells; mean $\pm$ SEM, $n = 3$). **C**, chemical structure of LBH589.

of caspase-3. These findings emphasize a cell-protective prosurvival role of mutant FLT3 in our cellular model and show that it counteracts the HDACi-triggered cell death. The chemical structure of LBH589 is presented in Fig. 1C.

LBH589 synergizes with the TKI cpd.102 to induce apoptosis of AML cells expressing FLT3-ITD

Next, we determined whether concurrent inhibition of FLT3-ITD with a TKI and exposure of FLT3-ITD-positive MV4-11 cells to LBH589 likewise results in enhanced cytotoxicity. From previous publications (17, 20), we knew that a dose of 30 nmol/L LBH589 very strongly affects MV4-11 cell survival and FLT3-ITD stability. For the purpose of combinatory treatment, we used this concentration as highest dose and explored the efficiency of 5 to 30 nmol/L LBH589 alone and in combination with TKI treatment. The doses of all TKI were defined on the basis of their differential potency to inhibit phosphorylation of FLT3-ITD analyzed by immunoblotting. We treated MV4-11 cells with the small-molecule FLT3 inhibitor cpd.102 (28), LBH589, or their combination. Single treatment with 10 nmol/L LBH589 or 300 nmol/L cpd.102 increased the ratio of apoptotic cells from around 9% in control cell populations to 25% or 17%, respectively (Fig. 2A). An additional decrease of cells in the G_2 and S-phases showed that the drugs also impaired cell proliferation.

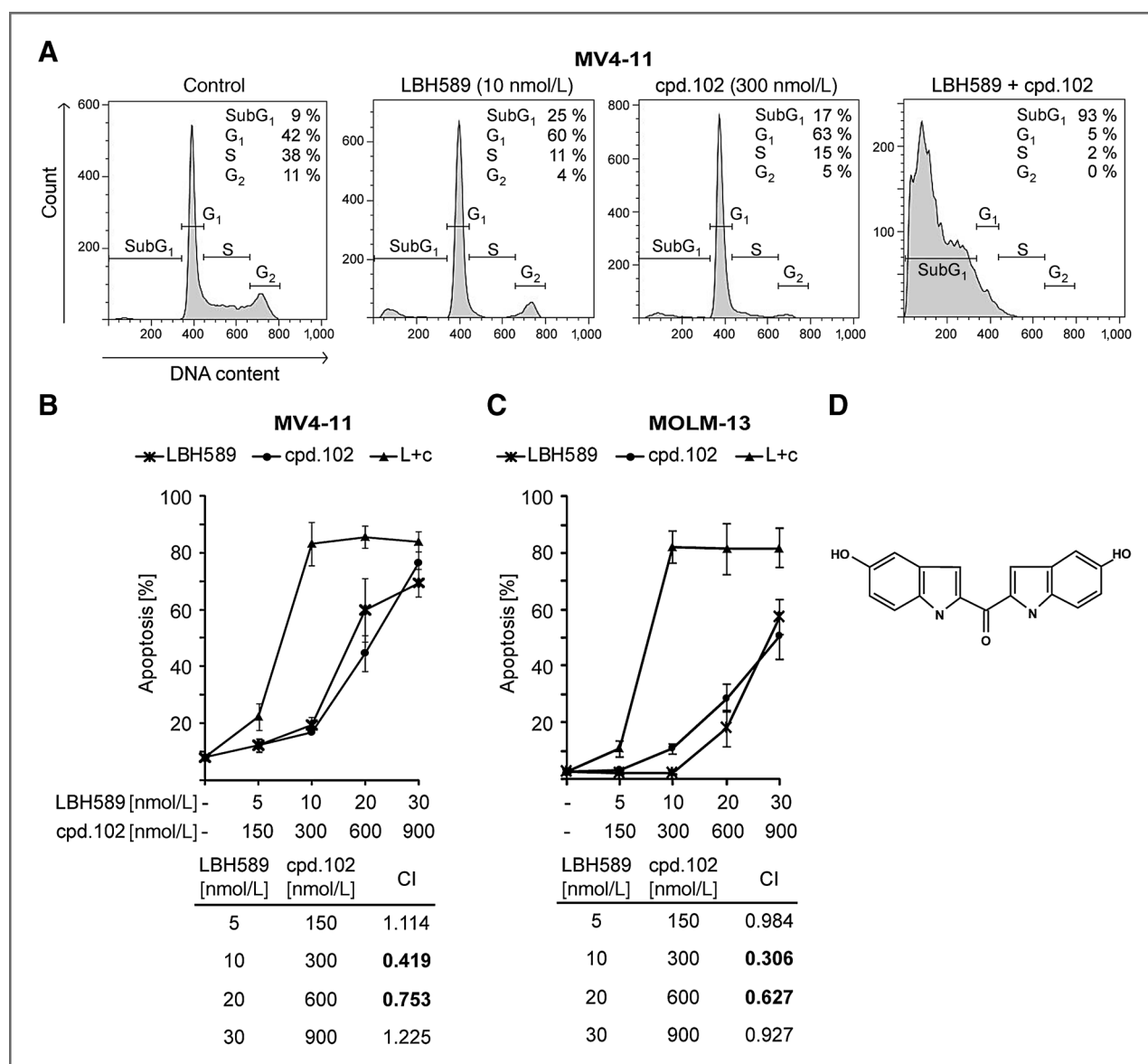


Figure 2. LBH589 and cpd.102 synergistically induce apoptosis of AML cells expressing FLT3-ITD. **A**, MV4-11 cells were treated with 10 nmol/L LBH589 and 300 nmol/L cpd.102 for 24 hours. Cell-cycle alterations were evaluated using PI-FACS analysis and a representative result is presented ($n = 4$). **B**, MV4-11 cells were treated with increasing concentrations of LBH589 (L) and cpd.102 (c) alone or in combination for 24 hours. Apoptosis rates were measured by PI-FACS analysis ($n = 4$, mean $\pm$ SEM). CI values for cotreatment with LBH589 and cpd.102 were calculated using the CalcuSyn Software. CI < 0.9 indicates synergism (bold). **C**, MOLM-13 cells (FLT3/FLT3-ITD) were treated and analyzed as described above (B). Mean $\pm$ SEM, $n = 3$. **D**, chemical structure of cpd.102.

Importantly, the combination of LBH589 and cpd.102 dramatically increased apoptosis rates reaching 93% (Fig. 2A and see Fig. 2B for statistical evaluation). These data show that concomitant inhibition of FLT3-ITD activity by the TKI in combination with LBH589 treatment results in a lethal effect in MV4-11 cells.

We then investigated whether the pro-apoptotic effects on AML cells that we achieved with our drug regimen displayed synergistic interactions. To test this, we exposed MV4-11 cells to varying concentrations of LBH589 and cpd.102, either as single treatment or in

combination. The median dose-effect analysis according to Chou and Talalay (31, 35) revealed CI values below 0.9 for 2 of 4 tested dose pairs, indicating drug synergism (Fig. 2B). While exposure of MV4-11 cells to very high doses of LBH589 or cpd.102 also induced significant rates of cell death, cotreatment markedly increased the numbers of apoptotic cells at significantly lower individual doses of each agent.

We further tested how HDACi/TKI regimens affect cells with a mutated and a wild-type allele of *FLT3* (MOLM-13; ref. 36). These cells also represent a model

for AMLs harboring *c-CBL* mutations supporting FLT3-dependent transformation (37). As in MV4-11 cells, the combinational treatment with LBH589 and cpd.102 induced high rates of apoptotic cell death of MOLM-13 cells. CI values <0.9 for 2 dose pairs tested show synergistic drug actions against MOLM-13 cells (Fig. 2C). The chemical structure of cpd.102 is presented in Fig. 2D.

Coadministration of LBH589 and cpd.102 reduces activation and stability of FLT3-ITD and STAT5

We further elucidated the molecular mechanisms underlying the enhanced cytotoxicity of HDACi/TKI regimens toward FLT3-ITD-positive cells. We incubated MV4-11 cells with various doses of LBH589 and cpd.102 and analyzed whether these drugs change the protein levels of critical signaling molecules. Consistent with our previous findings (20), LBH589 at concentrations of ≥ 20 nmol/L reduced the protein levels of FLT3-ITD. Degradation of the mutant receptor was accompanied by abrogation of STAT5 tyrosine phosphorylation (Fig. 3A), which is consistent with the FLT3-ITD-dependent STAT5 activation (7). Interestingly, when administered at ≥ 20 nmol/L, LBH589 also reduced total STAT5 protein levels. Concomitantly, caspase-3 activation and cleavage of its substrate PARP could be observed, confirming apoptosis

induction. Single treatment with cpd.102 at concentrations of 300 nmol/L and higher blocked FLT3-ITD phosphorylation at tyrosine 589 (pY589), an indicator site for receptor activation (Fig. 3A). At such doses, a complete loss of receptor activity correlated with caspase-3 activation, PARP cleavage, and degradation of FLT3-ITD and STAT5.

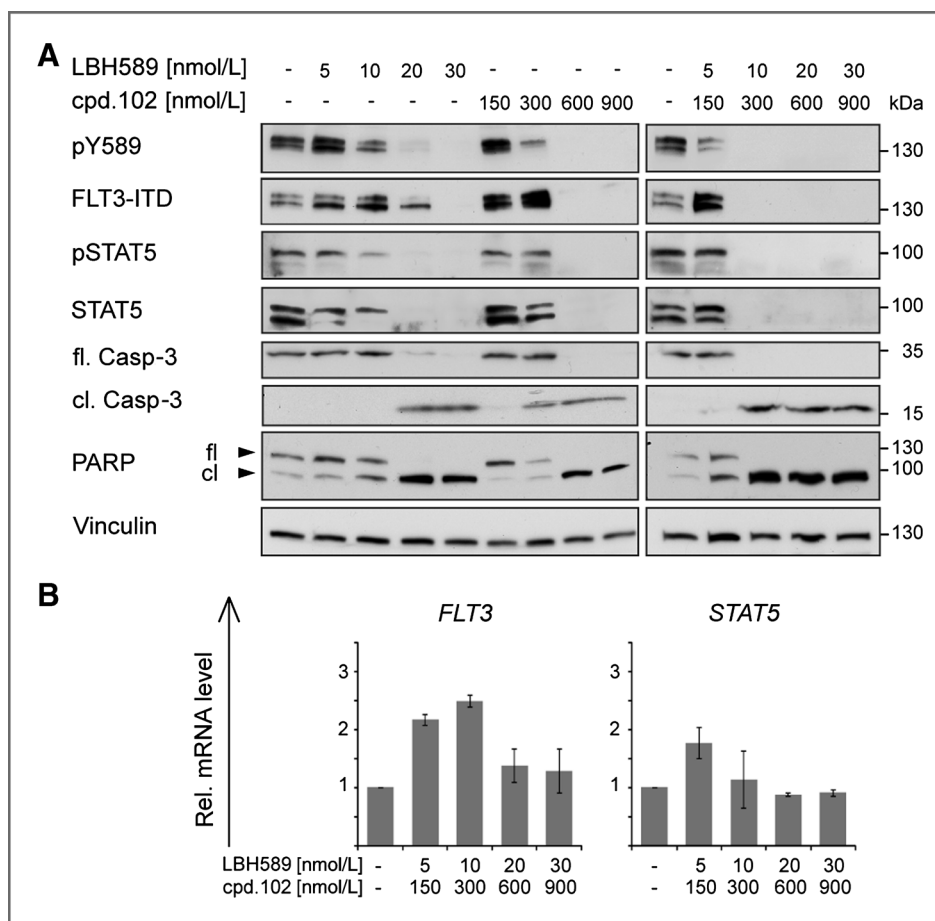
Compared with single drug administrations, the combination of LBH589 and cpd.102 evoked depletion of FLT3-ITD and STAT5 protein at low concentrations of both substances. This was accompanied by increased caspase-3 activation and PARP cleavage. These observations are consistent with the high levels of apoptosis measured in FACS analyses (Fig. 2B).

Real-time qPCR analyses indicated that the reduction of FLT3-ITD and STAT5 protein levels did not result from reduced *FLT3-ITD* and *STAT5* mRNA levels (Fig. 3B). Therefore, our results suggest that destabilization of these factors relies on molecular mechanisms operating at the protein level.

Caspases contribute to the degradation of FLT3-ITD and STAT5

Our experiments show that a loss of FLT3-ITD and STAT5 in cells exposed to TKI and HDACi correlates with

Figure 3. Coadministration of LBH589 and cpd.102 negatively affects activation and protein stability of FLT3-ITD and STAT5. **A**, MV4-11 cells were treated for 24 hours with LBH589 and cpd.102 as indicated, and cell lysates were subjected to immunoblot analyses. The antibody binding pSTAT5 recognizes pSTAT5a as well as pSTAT5b. **B**, mRNA levels of *FLT3* and *STAT5* in MV4-11 cells treated with LBH589 and cpd.102 were analyzed by real-time qPCR ($n = 2$; mean $\pm$ SEM). pY589, pFLT3-ITD (Y589); Casp, caspase; cl, cleaved; fl, full-length.



caspase-3 activation. Co-incubation of MV4-11 cells with the combination of LBH589 and cpd.102 in the presence of the cell-permeable pan-caspase inhibitor Z-VAD-FMK partially restored FLT3-ITD protein levels and almost completely stabilized STAT5, supporting a role of caspases for the loss of both proteins (Fig. 4A).

To directly assess whether STAT5 is a direct substrate of caspase-3, we overexpressed STAT5 in HEK293T cells and incubated cell lysates with bacterially expressed active caspase-3 *in vitro*. We found a significant decrease of STAT5 after incubation with active caspase-3. Tubulin levels remained stable upon this treatment illustrating the selectivity of the protease toward STAT5 (Fig. 4B). Caspase-3 hardly affected FLT3-ITD *in vitro* (data not shown). We therefore tested whether caspase-3 may have the capacity for degrading FLT3 in intact cells. We co-expressed FLT3 or FLT3-ITD together with caspase-3 or the alternative effector caspase-6 in HEK293T cells. To induce efficient caspase processing and activation in these cells, they were treated with the bacterial alkaloid staurosporine, which is widely used as a broad-range caspase activator and inducer of apoptosis. We found that the amount of caspase-3 correlated with reduction of FLT3-ITD and FLT3 levels. Protein levels of endogenously expressed tubulin and cotransfected GFP (as internal transfection control) were not affected, affirming protease specificity. In contrast, caspase-6 did not cleave FLT3-ITD or FLT3 (Supplementary Fig. S3). The fact that both FLT3 and FLT3-ITD can be degraded by caspase-3 suggests that

FLT3-ITD reduction in the LBH589/TKI-sensitive cell line MV4-11 is not intrinsic to ITD mutation but due to stronger apoptosis and caspase activation. In summary, these data suggest that caspase-3 is involved in the proteolytic cleavage of FLT3 and STAT5 and reveal that STAT5 is a direct target for this protease.

Clinically relevant LBH589/TKI drug combinations synergistically cause apoptosis of FLT3-ITD-positive AML cells

Our data show that coadministration of LBH589 and cpd.102 synergistically induces apoptosis of AML cells. We tested whether similar effects are achievable by combining LBH589 with TKIs that are currently tested in clinical trials. We tested AC220 and PKC412, which have both achieved promising results in patients (8, 38, 39).

We determined the anti-leukemic potency of these TKIs alone and in combination with LBH589. We conducted FACS analysis and immunoblotting for the detection of programmed cell death. All tested dose pairs of LBH589 and AC220 generated synergistic cytotoxic effects against MV4-11 cells (Fig. 5A). We confirmed the synergism of LBH589 and AC220 in MV4-11 cells by Annexin V staining of apoptotic cells (Supplementary Fig. S4), and very similar synergistic drug interactions were observed in MOLM-13 cells (Fig. 5B). PKC412 concomitantly administered with LBH589 also induced apoptosis of MV4-11 cells in a synergistic range (Fig. 5C). In comparison to PKC412, the FLT3-ITD-specific agent AC220 synergistically interacted with the HDACi across a broader dose range and already at very low nanomolar concentrations.

To validate the activity of LBH589 in combination with these TKIs in our cellular setting, we probed immunoblots with an antibody specifically recognizing FLT3 phosphorylated at Y589. We could confirm that both AC220 and PKC412 inhibited FLT3 phosphorylation (Fig. 5D and E). Like cpd.102, AC220 reduced FLT3-ITD protein levels (Fig. 5D), but PKC412 hardly destabilized FLT3-ITD (Fig. 5E). Compared with single treatments, concurrent exposure of MV4-11 cells to TKIs combined with LBH589 more strongly activated caspase-3 and caused depletion of FLT3-ITD and STAT5. Highest degree of synergism of LBH589 + TKI was achieved when single application of LBH589 or AC220 had not fully induced degradation of FLT3-ITD. The chemical structures of AC220 and PKC412 are presented in Fig. 5F and G, respectively.

To assess the specificity of LBH589/AC220 regimens for apoptosis induction, RS4-11 cells expressing the wild-type FLT3 receptor were used. In these cells, the combinatory treatment caused a significantly lesser increase in the apoptotic cell fraction and TKIs were ineffective (Fig. 6A and data not shown). Interestingly, the FLT3-ITD-negative cell lines K562 and U937 as well as normal PBMCs from 2 healthy donors were largely refractory to this drug combination when compared with MV4-11 or MOLM-13 cells expressing FLT3-ITD (Fig. 6A). The exclusive sensitivity of FLT3-ITD expressing cells was also seen

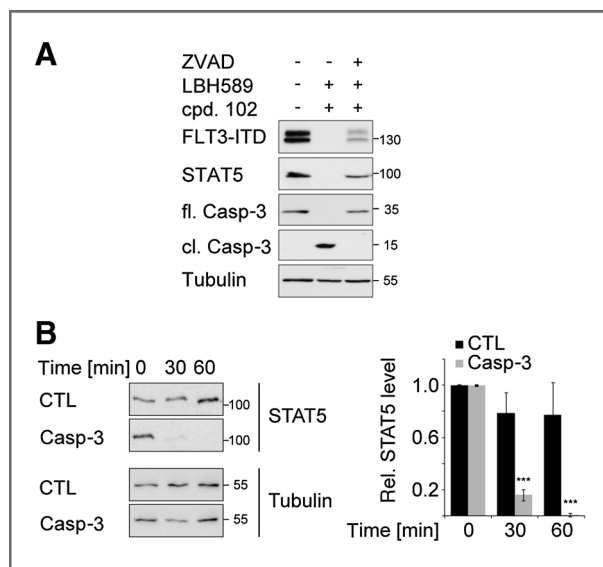


Figure 4. Caspases contribute to the degradation of FLT3-ITD and STAT5. **A**, MV4-11 cells were untreated (–) or treated (+) for 1 hour with the pan-caspase inhibitor Z-VAD-FMK (ZVAD, 50 μ mol/L) before incubation with LBH589 (30 nmol/L) and cpd.102 (900 nmol/L) for 24 hours. Protein levels were analyzed by immunoblotting. **B**, STAT5 was expressed in HEK293T cells. Their lysate was incubated with *E. coli* lysates with or without (CTL) bacterially expressed active caspase (Casp)-3 for up to 60 minutes. Protein levels were determined by immunoblotting and quantified by densitometric analysis ($n = 3$, mean $\pm$ SEM; ***, $P < 0.001$). cl., cleaved; fl., full-length.

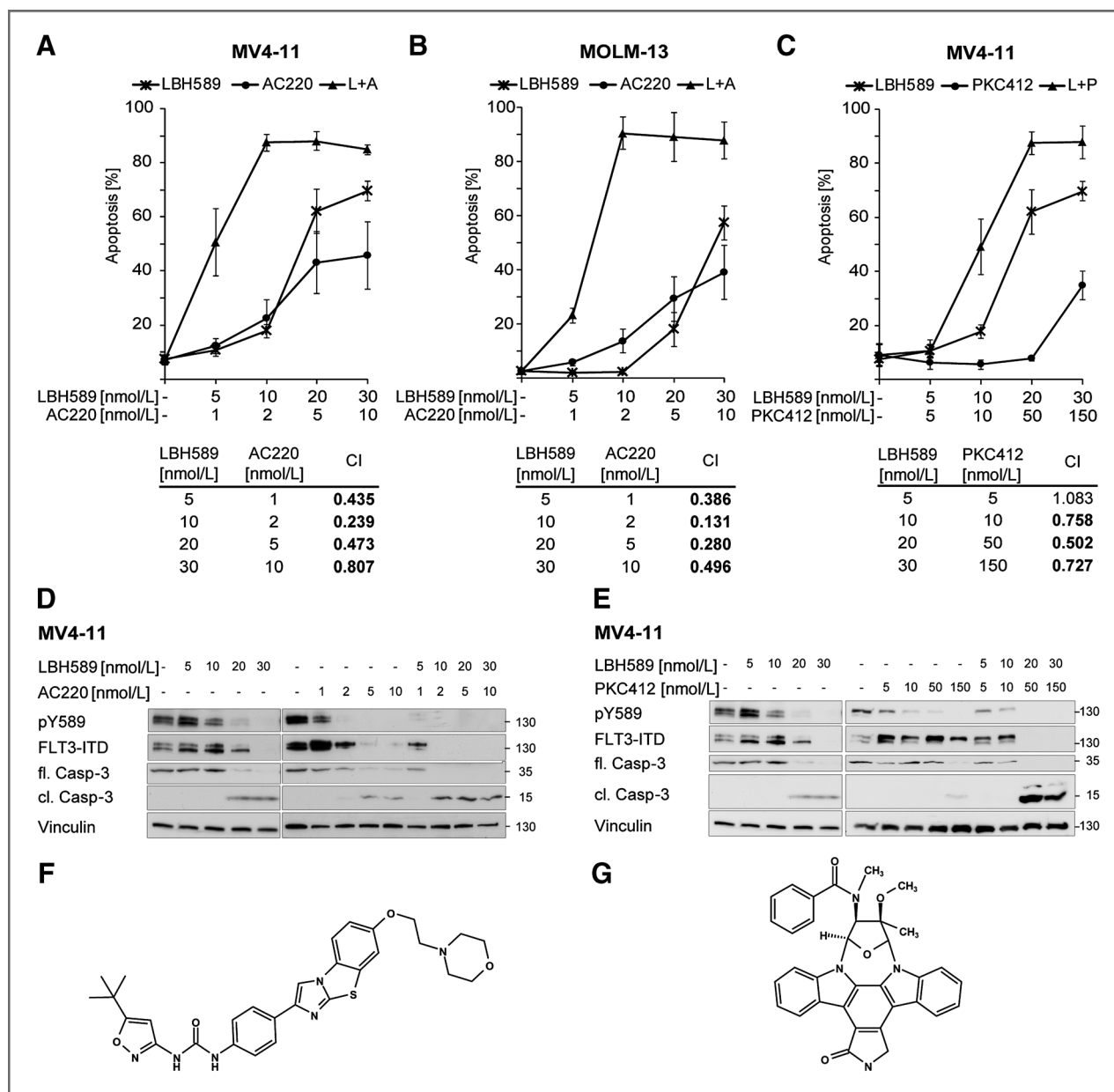


Figure 5. Combinations of LBH589 and AC220 or PKC412 synergistically induce apoptosis of AML cells. **A**, MV4-11 cells were treated with increasing concentrations of AC220 (A) and LBH589 (L) for 24 hours. Cells were tested for apoptosis rates by PI-FACS analysis ($n = 3$, mean $\pm$ SEM). CI < 0.9 indicates synergism (bold). **B**, MOLM-13 cells were treated and analyzed as in **A**. $n = 3$. **C**, MV4-11 cells were treated with PKC412 (P) and LBH589 at increasing concentrations. Cells were analyzed as described in **A**. $n = 3$, mean $\pm$ SEM. **D**, MV4-11 cells were treated with LBH589 and AC220 as described in **A**. Protein expression of these cells was analyzed by immunoblotting. **E**, MV4-11 cells were treated with PKC412 and LBH589 as described in **C**. Lysates were analyzed by immunoblotting. **F**, chemical structure of AC220. **G**, chemical structure of PKC412. cl, cleaved; fl, full-length.

at the level of STAT5 degradation. While LBH589/AC220 treatment efficiently decreased STAT5 levels in MV4-11 cells already at low doses, STAT5 was more stable or even unaffected in all other cell lines and PBMCs that were treated equally (Supplementary Fig. S5). To determine the effect of LBH589/AC220 treatment on growth factor-dependent cells, we analyzed interleukin (IL)-3-dependent Ba/F3 cells. Survival analyses revealed resistance of Ba/F3 cells to LBH589/AC220 combinations in the pres-

ence of IL-3, whereas oncogene addition in Ba/F3 cells stably transfected with FLT3-ITD conveyed susceptibility to LBH589/AC220 combinations (Supplementary Fig. S6). These findings provide further evidence that this treatment selectively targets cells expressing constitutively active FLT3-ITD.

During therapy, inhibitor concentrations are not constantly high due to plasma protein binding, segregation, and metabolism (13). To achieve conditions resembling

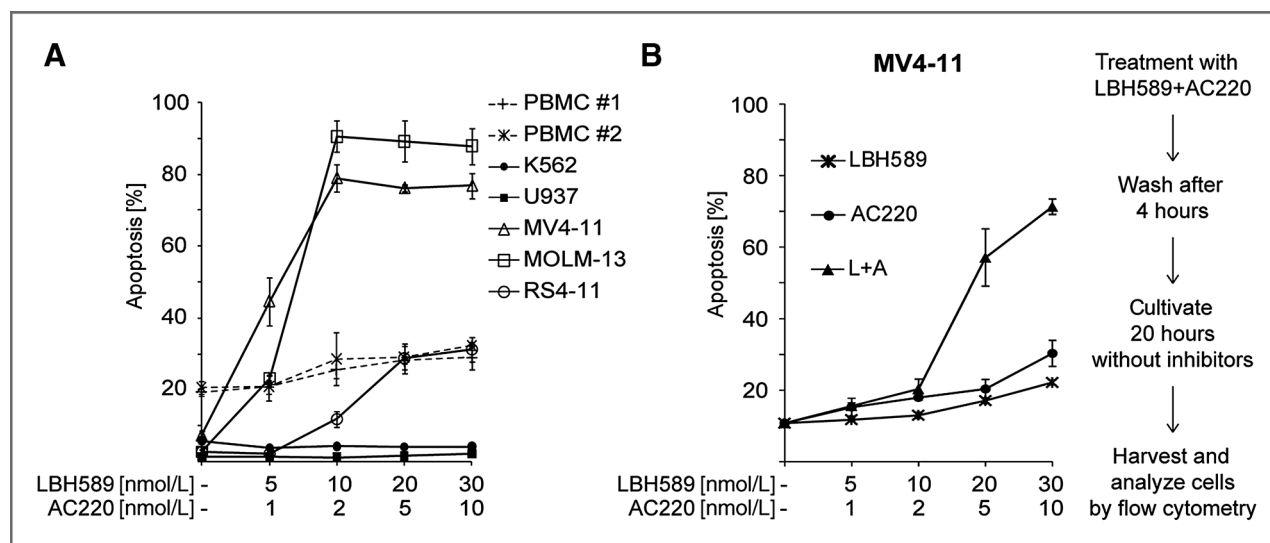


Figure 6. LBH589/AC220 regimens selectively affect FLT3-ITD-expressing cells. A, MV4-11, MOLM-13, RS4-11, U937, and K562 cells as well as PBMCs were treated with increasing concentrations of AC220 plus LBH589 for 24 hours. Cells were tested for apoptosis rates by PI-FACS analysis (mean $\pm$ SEM, $n = 3$). B, MV4-11 cells were treated with increasing concentrations of LBH589, AC220, or a combination of both. After 4 hours, cells were washed and subsequently incubated without inhibitors for further 20 hours. Apoptosis rates were measured by PI-FACS analysis (mean $\pm$ SEM, $n = 3$).

short-term drug exposure, we incubated MV4-11 cells with both agents for 4 hours, washed off the inhibitors, and cultivated the cells for another 20 hours. Such treatment with LBH589 or AC220 alone did not generate a considerable increase in the apoptotic cell fraction after 24 hours. Strikingly, a 4-hour exposure to a combined HDACi/TKI regimen was sufficient to induce similar rates of apoptotic cell death as continuous treatment for 24 hours (Fig. 6B).

Discussion

FLT3-ITD mutations are frequent in patients with AMLs (40) and are associated with less favorable prognoses and increased risk for relapse (41–43). Development of a therapy specifically targeting FLT3-ITD-positive AML cells may particularly benefit these patients. It is possible that such strategies efficiently target leukemic blasts and have increased therapeutic windows. While several inhibitors of the FLT3 receptor are under investigation in clinical trials (8, 15, 38, 39), FLT3 TKI monotherapy will likely not suffice for curing the disease. In the present study, we evaluated the effects of the combinatorial treatment with the HDACi LBH589 and different FLT3 inhibitors on AML cells. All tested combinations of these substances resulted in synergistic apoptosis induction.

A recent study highlights the relevance of mutant FLT3 as a driver of AML and hence as an important therapeutic target (6). Moreover, several TKIs against FLT3 have reached phase III clinical trials (12, 14, 44). We could confirm high efficiency of the specific FLT3 inhibitor AC220 and its superiority over cpd.102 and PKC412. At present, AC220 is considered the most promising TKI for AML therapy (12, 13), but inhibitor-resistant secondary

mutations were, for example, selected in 8 of 8 patients under AC220 treatment (6). One of the basic goals of combinatorial therapies is to avoid secondary resistances that frequently cause a relapse of the disease (14). In this context, we noted that sublethal doses of TKIs increase FLT3-ITD levels. This finding is consistent with our previous results showing that phosphorylation triggers proteasomal degradation of FLT3-ITD (20). The advantage of the combinatory treatment with LBH589 and TKIs presented here at least partially relies on the ability of this HDACi to induce proteasomal degradation of FLT3-ITD (20, 45). Curiously, this cannot be achieved by other clinically relevant HDACi like suberoylanilide hydroxamic acid, which is about one log less potent than LBH589 (19). The LBH589-induced reduction of FLT3-ITD protein efficiently complements the inhibition of receptor activity by FLT3 inhibitors. Our data suggest that inhibition and loss of FLT3-ITD tip the balance toward cell death in AML cells that rely on FLT3-ITD signaling. The resulting principle, to hit AML blasts *hard and early*, could have an increased efficiency in eliminating leukemic blasts from the blood and bone marrow and could also counteract the development of TKI resistance. Therefore, such treatment might show enhanced clinical efficacy.

AC220 turned out to be the most efficient TKI in combination with LBH589. We obtained very high rates of cell death with the combination of low single doses of LBH589 and TKI. This is noteworthy as the aim of combining agents is to improve therapy and to reduce toxicity (e.g., with lower drug concentrations or specific administration schedules). Our experiments with normal PBMCs show that drug concentrations causing more than 80% apoptosis in FLT3-ITD-positive MV4-11 cell cultures did not affect normal cells. This observation provides a

hint that blood cells expressing wild-type FLT3 could be spared by low-dose combinatory treatment with LBH589 and TKI. The superior action of LBH589 plus TKI is evident over a range of low nanomolar concentrations. Of note, LBH589/TKI combinations efficiently cause cell death of FLT3-ITD-positive AML cells, even when such cells are exposed to both agents for short-term (followed by a wash-out and growth without agents). Conversely, individual administration of the single compounds proved ineffective. In this assay, LBH589/TKI administration probably evokes immediate, irreversible changes in signaling and gene expression. Experiments are underway to decipher these pathways and their relevance for apoptosis.

In many AMLs, STAT5 is a constitutively activated oncogenic driver and therefore a potential target (46, 47). This transcription factor is also important for FLT3-ITD-driven malignancy (7, 34, 46, 47). We present that the most potent pro-apoptotic combinations of HDACi and TKI reduce STAT5. Hence, destabilization of STAT5 and FLT3-ITD could be main advantages of the LBH589/TKI combinatory treatments. In addition, depletion of these factors could possibly serve as a predictive marker for the therapeutic efficacy of these agents.

We could show that caspases are involved in the degradation of FLT3-ITD, as well as of STAT5. Our data further suggest that STAT5 is a direct caspase-3 substrate. Thus, besides proteasomal degradation of active FLT3 (20), the LBH589 + TKI-induced depletion of these oncoproteins is partially mediated by caspases. Our current understanding of the molecular actions induced by the combination therapy can be summarized as follows: LBH589 induces the proteasomal degradation of constitutively active FLT3-ITD (20). Simultaneously, the FLT3 inhibitor blocks catalytic activity of the receptor. At low concentrations, each single drug is not able to completely inactivate FLT3 in a sustained manner. However, both processes cooperatively increase receptor degradation while at the same time block its activity and subsequent signaling events. The elimination of essential FLT3-ITD-mediated growth and survival signals results in strong activation of caspases and induction of apoptosis of AML cells. Moreover, activated caspase-3 cleaves STAT5 and possibly contributes to the degradation of FLT3-ITD. Our observation that already short-term combinatory treatment effectively induces apoptosis argues for a positive feedback mechanism with a point-of-no-return.

In summary, we reveal synergistic interactions of the clinically relevant HDACi LBH589 and 3 TKI (AC220, cpd.102, PKC412). We show that strong induction of apoptosis occurs even when low doses of the single substances are used in combination. HDACi plus TKI induce inhibition and degradation of FLT3-ITD and block downstream signaling pathways for AML cell survival. Our data suggest that molecular mechanisms of this synergism rely on the depletion of FLT3-ITD and the caspase-mediated cleavage of STAT5. Apparently, combinatory HDACi/TKI application targets several aspects of oncogene addiction characterizing FLT3-ITD-positive AMLs. Such drug combinations could therefore become a major benefit for a currently difficult to treat subgroup of patients with AMLs.

Disclosure of Potential Conflicts of Interest

G. Bug has received honoraria and travel grants and is a consultant/advisory board member of Novartis Pharma GmbH. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: K. Pietschmann, H.A. Bolck, M. Buchwald, K. Spiekermann, G. Bug, T. Heinzel, O.H. Krämer

Development of methodology: K. Pietschmann, H.A. Bolck, M. Buchwald, F.-D. Böhmer

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Bug, O.H. Krämer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Pietschmann, H.A. Bolck, G. Bug, F.-D. Böhmer, O.H. Krämer

Writing, review, and/or revision of the manuscript: K. Pietschmann, H.A. Bolck, H. Polzer, K. Spiekermann, G. Bug, T. Heinzel, F.-D. Böhmer, O.H. Krämer

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Pietschmann, H.A. Bolck, S. Spielberg, H. Polzer, G. Bug, O.H. Krämer

Study supervision: O.H. Krämer

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