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Role and regulation of MicroRNAs targeting BTK in acute myelogenous leukemia

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The Brutons' tyrosine kinase, BTK becomes constitutively activated by the Fms-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) or the toll like receptor 9 (TLR9) in acute myelogenous leukemia (AML)^{1–3}. This in turn, initiates a signaling cascade that facilitates survival and proliferation^{1–3}. Pharmacological inhibition of BTK using ibrutinib, a potent and irreversible kinase inhibitor of BTK, or genetic knockdown of BTK cause selective toxicity in AML bearing FLT3-ITD² and initiated its clinical evaluation as a single agent and in combinations with other chemotherapy for AML⁴. However, one recent study identified that ibrutinib also targeted FLT3-ITD⁵ making it necessary to determine the role of BTK in the survival of AML cells with differing FLT3 status. Ectopic expression of control siRNA (Ctrl) and three different BTK siRNAs in OCI-AML3 (FLT3-WT) and MV4-11 (FLT3-ITD) cells (Fig. 1A) resulted in a knock-down of BTK protein and mRNA ($p < 0.0001$, Supp. Fig. 1A). siBTK effectively compromised the proliferation and survival of both OCI-AML3 and MV4-11 cells in colony forming assays (Mean CFU inhibition: 40-55% in OCI-AML3, $p < 0.05$; 28-43% for MV4-11, $p < 0.01$) (Fig. 1A, graphs) thus establishing that BTK was a relevant therapeutic target in AML cells irrespective of FLT3-ITD status.

Since BTKi's preferentially targeted FLT3-ITD positive AML, we sought to identify mechanisms capable of targeting BTK irrespective of their FLT3 status. MicroRNAs are gene regulators that regulate target abundance at a post transcriptional level⁶. We recently identified that BTK was regulated by a key signature of microRNA (miR-147b, miR-210, miR-425, miR-1253, miR-4269 and miR-4667) in B malignancies⁷ (Supp. Fig. 1B). Ectopic expression of miR-147b, miR-210, miR-425, miR-1253, miR-4269 and miR-4667 in OCI-AML3 or MV4-11 cells demonstrated that each of these miRs with the exception of miR-1253 effectively decreased BTK protein when compared to levels in cells expressing the scrambled (Scr) oligonucleotide (OCI-AML3: miR-147b, miR-425, miR-4269 and miR-4667: $p < 0.0001$, miR-210: $p < 0.001$) (Fig. 1B, upper panels). Flow cytometry analysis provided independent confirmation that BTK protein expression was significantly down-

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regulated by each of these miRs except for miR-1253 (Supp. Fig. 1C). Significantly, expression of the BTK targeting miRNA in OCI-AML3 showed that miR-147b, miR-210, miR-425, miR-4269 strongly reduced the proliferative potential of OCI-AML3 cells in comparison to cells expressing scrambled miRNA (Fig. 1B, lower panel graphs) (Mean CFU inhibition: 16-40% for miRNA, miR-147b ($p<0.01$), miR-210: $p<0.001$, miR-425 and miR-4269: $p<0.0001$). Similar results were obtained in MV4-11 cell line (Fig. 1B, lower panel graphs), where each miR induced a marked reduction in colony number as compared to controls (Mean CFU inhibition: 37-60% for miRNA, miR-147b, miR-425 and miR-4269: $p<0.01$, miR-210 and miR-4667: $p<0.001$, miR-1253: $p<0.0001$). Finally, we evaluated whether miR-147b, miR-210, miR-425-5p, miR-1253, miR-4269, and miR-4667 targeted BTK directly, and found that ectopic expression of miR-147b ($p<0.0001$), miR-210 ($p<0.05$), miR-425-5p ($p<0.0001$), miR-4269 ($p<0.0001$) and miR-4667 ($p<0.01$) in OCI-AML3 cells suppressed the activity of a *Renilla luciferase* construct containing BTK 3' UTR by 10-30% at 48h (Fig. 1C, black histograms) when compared to the ability of a miR (mir-29b) not predicted to target BTK or to luciferase constructs bearing deletions at each binding site (Fig. 1C, white histograms).

When we quantitated the expression of the BTK-targeting miRNA in AML we found that miR-147b ($p<0.001$), miR-210 ($p<0.01$), miR-4269 and miR-4667 ($p<0.0001$) were significantly under-expressed in AML patients ($n=8$ FLT3-WT, $n=4$ FLT3-ITD) as compared to CD34+ cells isolated from healthy donors (Fig. 2A, left panel). In B cell malignancies, HDAC inhibition led to an induction in the expression of the BTK targeting miRs with reciprocal decreases in BTK expression and an inhibition of its pro-survival signaling⁷. Additionally it synergized with Ibrutinib to dually target BTK and result in loss of BTK signaling and leukemia cell survival in primary samples as well as in mouse models⁷. While HDAC inhibitors were inactive in CLL⁸, they are therapeutically active in AML and induced responses of between 10-30% as a single agent and higher when used in combination with other chemotherapeutic regimens^{9, 10}. When blasts from 13 AML patients ($n=8$, FLT3-WT and $n=5$, FLT3-ITD) were exposed to 10nM of the pan-deacetylase inhibitor panobinostat (PS) for 48h, there was an induction in the expression of miR-147b, miR-210, miR-4269, and miR-4667 by 24 h (miR-147b and miR-4667: $p<0.01$, miR-210: $p<0.001$, miR-4269: $p<0.05$) (Fig. 2A, right panel) which was accompanied by a time-dependent depletion of total BTK protein (Fig. 2B, upper panel) in most AML blasts (Fig 2B-lower panel, left graph), decreases in pBTK, as well as an inhibition of BTK signaling via p-AKT and pERK (Fig. 2B, upper panel). HDAC inhibition resulted in a modest decrease in the levels of the BTK mRNA in 4 of 9 samples tested (Fig. 2B, center graph) and induced robust cell death in AML patients (9.6-92% apoptosis, Mean: 55%, Fig. 2B, right graph). These results indicated that a set of HDAC regulated miRs targeted BTK effectively in both FLT3-WT and FLT3-ITD positive AML.

Next, we determined whether HDAC inhibitors would cooperate with kinase inhibitors of BTK to synergistically abolish BTK signaling and induce death in AML cells. Exposure to Ibrutinib as a single agent inhibited the kinase activity of BTK (measured by the auto-phosphorylation on Y223) in OCI-AML3 cells (Fig. 2C, upper panel) and MV4-11 cells (Supp. Fig. 2A) but did not affect total BTK protein, expression of the BTK-targeting miRs (Fig 2C, middle panels and Supp. Fig 2B) or abolish its downstream signaling in either cell

line (Fig. 2C, upper panel and Supp. Fig. 2A). Exposure to the pan HDAC inhibitor abexinostat as a single agent induced both miR-210 and miR-4667 in OCI-AML3 cells (Fig. 2C, middle panel) and induced miR-4269 and miR-4667 in MV4-11 cells (Supp. Fig. 2B). This was accompanied by decreases in total BTK protein, p-BTK and an attenuation of BTK signaling in OCI-AML3 (Fig. 2C) and MV4-11 cells (Supp. Fig. 2A). In cells exposed to the combination of abexinostat and Ibrutinib, the induction of miR-210 and miR-4667 in OCI-AML3 cells and of miR-4269 and miR-4667 was not significantly different from HDACi alone (Fig. 2C, middle panel and Supp. Fig. 2B). However, the combination exhibited a dual targeting of BTK, where Ibrutinib inhibited the kinase function of BTK and HDAC inhibition targeted total BTK in both OCI-AML3 and MV4-11 cells which was reflected in a potent inhibition of BTK signaling via p-AKT and p-ERK. (Fig. 2C and Supp. Fig. 2A).

Finally we tested whether HDAC inhibitors synergized with Ibrutinib in compromising survival of AML cells using two different HDAC inhibitors (abexinostat and PS). The combination of abexinostat and Ibrutinib induced a greater-than-additive cytotoxicity compared to Ibrutinib alone in OCI-AML3 (Supp. Fig. 2C, 1 μ M Ibrutinib vs 0.2 μ M abexinostat+Ibrutinib: $p<0.05$, 1 μ M Ibrutinib vs 0.4 μ M abexinostat+Ibrutinib: $p<0.0001$) but not when compared to abexinostat alone. However, this combination was synergistic in activating cell death in comparison to either single agent in MV4-11 cells (Supp. Fig. 2D, 1 μ M Ibrutinib vs 0.2 μ M abexinostat+Ibrutinib: $p<0.05$, 1 μ M Ibrutinib vs 0.4 μ M abexinostat+Ibrutinib: $p<0.001$, 0.4 μ M abexinostat vs 0.4 μ M abexinostat+Ibrutinib: $p<0.05$). When we tested the effect of PS and Ibrutinib on the proliferative capacity of AML cells, we found that exposure to Ibrutinib had little effect on the proliferative potential of either cell line whereas exposure PS significantly suppressed the formation of colonies derived from both cell lines (OCI-AML3, Fig. 2C; 1nM PS vs ctrl or Ibrutinib: $p<0.05$, and MV4-11, Fig. 2C 1nM PS vs ctrl or Ibrutinib: $p<0.01$ and $p<0.05$ respectively). The combination of PS and Ibrutinib synergized to decrease survival and colony formation in both OCI AML3 and MV4-11 cells (OCI-AML3, Fig. 2C; 1nM PS +Ibrutinib vs ctrl or Ibrutinib: $p<0.0001$, 1nM PS+Ibrutinib vs 1nM PS: $p<0.05$ and MV4-11, Fig. 2C; 1nM PS+Ibrutinib vs ctrl or Ibrutinib: $p<0.0001$ and 1nM PS+Ibrutinib vs 1nM PS: $p<0.0001$).

In conclusion, our data demonstrate a strategy that allows targeting of BTK in AML independent of their FLT3 status and provide a rationale for the clinical evaluation of histone deacetylase inhibitors in combination with Ibrutinib for AML patients in order to maximize the therapeutic potential of Ibrutinib in AML cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. BTK is a relevant therapeutic target in AML and is effectively targeted by several miRNA

(A) Upper panels: Western blot analysis of BTK in OCI-AML3 and MV4-11 transiently nucleofected with 150 nM of three validated stealth siRNAs against BTK or Negative Control (Ctrl). Lower panels: Colony formation assay of OCI-AML3 and MV4-11. Cells were transfected with 150 nM siRNA and after 24-48h plated in MethoCult™ for 7-14 days before being assayed for colony formation using a GelCount scanner (Oxford Optronix). (B) Upper panels: Western blot analysis of BTK in OCI-AML3 and MV4-11 transiently nucleofected with 300 nM mimics of miR-147b, miR-210-3p, miR-425-5p, miR-1253, miR-4269, and miR-4667-3p or Negative Control #1 (Scr) and analyzed after 72h. Relative BTK protein levels in OCI-AML3 transiently nucleofected with 300 nM mimics at 72h (comparison to Scr). Lower panels: Colony formation assay of OCI-AML3 and MV4-11. Cells were transfected with 300 nM mimics, and after 24-48h plated in MethoCult™ for 7-14 days before being assayed for colony formation using a GelCount scanner (Oxford Optronix). (C) Relative luciferase activity of psiCHECK2-BTK and psiCHECK2-BTK_mut measured in OCI-AML3 cell line transfected with 100 nM mimics of miR-147b, miR-210-3p, miR-425-5p, miR-1253, miR-4269, and miR-4667-3p or the non-BTK targeting miR, miR-29b. Luciferase activity at 48h was normalized to miR-29b. Black bars: cells transfected with psiCHECK2-BTK; white bars: cells transfected with psiCHECK2-BTK_mut. Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Values are the mean $\pm$ SEM from at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ unpaired t-test, two-tailed.

Figure 2. Effect of treatment with histone deacetylase (HDAC) inhibitors (panabinoestat or abexinostat) alone or in combination with Ibrutinib in AML cell lines and blasts

(A) Expression levels of miR-147b, miR-210-3p, miR-425-5p, miR-1253, miR-4269, and miR-4667-3p were evaluated in 8-12 AML samples. snoRNA U48 was used as endogenous control and the resulting $2^{-\Delta\Delta C_t}$ values were normalized to the mean of 7 CD34+ cells isolated from healthy donors. Induction of miRNA targeting BTK in 13 AML patient samples upon 24h treatment with 10 nM panabinoestat (PS). snoRNA U48 was used as endogenous control and the resulting $2^{-\Delta\Delta C_t}$ values were normalized against those from the same patient before treatment. (B) Upper panel: Immunoblots of BTK, p-BTK, ERK, p-ERK, AKT and p-AKT in blasts from two representative AML patients (one FLT3-WT and one FLT3-ITD positive) at 0-12-24-48 hours upon treatment with 10 nM PS. Lower panel: Relative BTK (left panel) and BTK mRNA (middle panel) expression levels and percentage of Annexin V positive (apoptotic) cells (right panel) in 14 AML patients treated with 10nM PS. Expression values at 12-24h were normalized to the untreated samples (0h). (C) Upper panel: cImmunoblots analysis of H3K9Ac, BTK, p-BTK, ERK, p-ERK, AKT and p-AKT in OCI-AML3 treated with 0.2 μM abexinostat, 1 μM Ibrutinib for 2h followed by washout, or a combination of 0.2 μM abexinostat with prior 2 hr exposure to 1 μM Ibrutinib with

washout.) Middle panel: Cells were harvested 8-16-24-36-48h after treatment with abexinostat. Relative expression levels of miR-210-3p and miR-4667-3p in OCI-AML3 upon 16-24-36h treatment with 0.2 μ M abexinostat, 1 μ M Ibrutinib for 2h followed by washout, or a combination of 0.2 μ M abexinostat with prior 2 hr exposure to 1 μ M Ibrutinib with washout. Expression levels were normalized to the untreated samples (0h.) Lower panel: Colony formation assay of OCI-AML3 and MV4-11 exposed to 1 nM PS, 1 μ M Ibrutinib for 2h followed by washout, or a combination of 1 nM PS with prior 2 hr exposure to 1 μ M Ibrutinib with washout. Values are the mean $\pm$ SEM from at least three independent experiments. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 unpaired t-test, two-tailed.