Synergistic Targeting of AML Stem/Progenitor Cells With IAP Antagonist Birinapant and Demethylating Agents

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Background  Acute myeloid leukemia (AML) therapy has limited long-term efficacy because patients frequently develop disease relapse because of the inability of standard chemotherapeutic agents to target AML stem/progenitor cells. Here, we identify deregulated apoptotic components in AML stem/progenitor cells and investigate the individual and combinatorial effects of the novel inhibitor of apoptosis (IAP) protein antagonist and second mitochondrial-derived activator of caspases (SMAC) mimetic birinapant and demethylating epigenetic modulators.

Methods  Protein expression was measured by reversed-phase protein array in AML patient (n = 511) and normal (n = 21) samples and by western blot in drug-treated cells. The antileukemic activity of birinapant and demethylating agents was assessed in vitro and in an in vivo AML mouse xenograft model (n = 10 mice per group). All statistical tests were two-sided.

Results  Compared with bulk AML cells, CD34^+38^- AML stem/progenitors expressed increased cIAP1 and caspase-8 levels and decreased SMAC levels (one-way analysis of variance followed by Tukey's multiple comparison test, \( P < .001 \)). Birinapant induced death receptor-/caspase-8–mediated apoptosis in AML cells, including in AML stem/progenitor cells, but not in normal CD34^+ cells. Demethylating agents modulated extrinsic apoptosis pathway components and, when combined with birinapant, were highly synergistic in vitro (combination index < 1), and also more effective in vivo (\( P < .001 \), by Student t test, for the median survival of birinapant plus 5-azacytidine vs birinapant alone or vs controls).

Conclusions  cIAP1, SMAC, and caspase-8 appear to play a role in AML stem cell survival, and synergistic targeting of these cells with birinapant and demethylating agents shows potential utility in leukemia therapy.


Activation of the intrinsic apoptosis pathway by chemotherapeutic agents is the primary treatment strategy for patients with acute myeloid leukemia (AML). Nevertheless, most patients ultimately relapse because of the persistence of disease-driving AML stem/progenitor cells that are refractory to chemotherapy (1,2). Inhibitor of apoptosis (IAP) proteins are important for regulating cell survival. They are expressed in various malignant cells, and this corresponds with poor treatment outcomes (3,4).

IAP proteins have only recently received attention as therapeutic targets. We have previously identified survivin and the X-linked inhibitor of apoptosis protein (XIAP) as potential targets for AML therapy (5–7). Unfortunately, only antisense oligonucleotide (ASO) for XIAP is available. Interestingly, although the initial results seemed promising (8), XIAP ASO trials demonstrated little or no impact on cancer progression (9). However, we demonstrated that XIAP ASO induced apoptosis preferentially in AML stem/progenitor cells (10). IAP proteins also modulate NFkB activity, which is constitutively active in AML cells (11), which can inhibit the intrinsic apoptosis pathway (12–14).

IAP proteins are antagonized by second mitochondrial-derived activator of caspases (SMAC) proteins (15,16). SMAC mimetics developed previously induce degradation of IAP proteins, especially baculoviral IAP repeat-containing protein 2 (cIAP1), promote death receptor ligand-induced caspase-8–mediated apoptosis in malignant cells (13,17,18), and are less toxic to normal cells (19). Birinapant, a novel bivalent SMAC mimetic with high affinity for IAP proteins, has excellent pharmacokinetic/pharmacodynamics properties, and it is in clinical trials both as a single agent and in combination agent chemotherapy (20,21). However, the expression of cIAP1 (the main target of SMAC mimetics), caspase-8 (the target of cIAP1), SMAC (the cellular antagonist of IAPs), and the antileukemic effectiveness.
of birinapant against AML cells and AML stem/progenitors have not been investigated.

Currently and historically, antileukemia drugs are tested without consideration of the microenvironment in which leukemic cells reside. The bone marrow (BM) microenvironment plays critical roles in chemoresistance (22–24). AML cells, in particular AML stem/progenitor cells, are in close contact with mesenchymal stromal cells (MSCs) in a hypoxic environment (25), which makes them resistant to chemotherapy not only because of their cell-intrinsic mechanisms but also because of microenvironmental factors associated with low oxygen tension (eg, those associated with chemotherapeutic agent–induced reactive oxygen species production).

In this study, we first examined the expression of cIAP1, caspase-8, and SMAC in AML blasts, AML stem/progenitor cells, and normal CD34+ cells by reverse-phase protein array. We then evaluated the therapeutic potential of birinapant and its combinations with demethylating agents in AML cells under physiologically relevant conditions and in an in vivo AML xenograft mouse model. Here we report that birinapant is effective, alone and in combination with demethylating agents, against AML cells and AML stem/progenitor cells, which we believe are clinically relevant findings that will greatly impact the therapy of AML.

Methods

Cells, Cell Culture, and Treatments

BM or blood samples from AML patients (n = 14) and normal subjects (control subjects, n = 4) were acquired after informed consent according to the Declaration of Helsinki following protocols approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center. The patient characteristics are presented in Table 1. Mononuclear cells were purified using a Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) density-gradient centrifugation. Cells were treated with birinapant alone or in combination with other agents under standard, MSC coculture, hypoxic, or MSC coculture/hypoxic conditions. The NCI-60 cell lines were treated with birinapant in combination with other agents as described in the Supplementary Methods (available online). To determine 5-aza second sensitivity in cIAP1 knockdown cells, OCI-AML3 cells were transfected with cIAP1 small interfering RNAs (siRNAs) BIRC2z2, 3, 7, and 8, and control siRNAs (Qiagen, Germantown, MD) as previously described (26). 5-Aza was added at 0 and 48 hours. Apoptosis induction was determined at 96 hours. In parallel, cells were transfected with siRNAs and cell lysates were obtained at 48 hours for validation of target knockdown.

Table 1. Acute myeloid leukemia patient characteristics*

<table>
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<tr>
<th>Patient</th>
<th>Source</th>
<th>Blast %</th>
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<th>Cytogenetics</th>
<th>In vitro treatment</th>
<th>Annexin V assay</th>
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<td>BM</td>
<td>52</td>
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<td>Intermediate</td>
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<td>Bulk CD34-</td>
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<td>96</td>
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<td>Unfavorable</td>
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<tr>
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<td>Bulk CD34-</td>
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<tr>
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<td>97</td>
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* 5-Aza = 5-azacytidine; AML = acute myeloid leukemia; BM = bone marrow; DAC = decitabine; MDS = myelodysplastic syndrome; MPD = myeloproliferative disorder; PB = peripheral blood.
Statistical Analyses
One-way analysis of variance, followed by Tukey’s multiple comparison test, was used to compare levels of proteins in bulk, CD34+, and CD34+38− cells. The results were expressed as mean ± standard deviation (SD). The protein levels in blasts from 511 newly diagnosed AML patients and in CD34+ cells from 21 normal control subjects were compared by two-sample t test. Triplicate results using cell lines or AML patient samples are expressed as mean ± SD. Statistical differences in cell death between cells cultured under different conditions were determined using paired Student t test, and P values less than .05 were considered statistically significant. All statistical assessments were two-sided. The combination index (CI) was determined by Calcusyn (Cambridge, MA) software using the Chou-Talalay method (34) and is expressed as the mean of CI values obtained at the effective dose for 50, 75, and 90% of the treatment population (i.e., ED50, ED75, and ED90 respectively). A CI less than 1 is considered synergistic and a CI equal to 1 is considered additive.

Xenograft Studies in NOD/SCID IL2Rγ Null (NSG) Mice
Animal experiments were done in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center. Molm13 cells (0.5 × 10⁶) stably expressing a dual Renilla luciferase–GFP reporter were injected into the tail vein of 8- to 12-week-old NSG mice (Jackson Laboratory, Bar Harbor, ME). Starting on day 3, mice (n = 10 mice per group) were treated intraperitoneally with a vehicle (phosphate-buffered saline/50 mM citrate, pH 5.0), 5 mg/kg 5-Aza, 15 mg/kg birinapant, or both. 5-Aza was administrated daily for 5 days, and birinapant was administered twice a week for 4 weeks. For the combination, birinapant administration was started after 5-Aza had ended. Tumor burden was monitored using the IVIS-200 noninvasive bioluminescence in vivo imaging system (Xenogen, Hopkinton, MA) after injecting the mice with the luciferase substrate coelenterazine (Biotium, Hayward, CA). Circulating Molm13-GFP/Luc cells were determined by flow cytometric measurement of human CD45+ cells in blood samples (n = 3) from the mice. cIAP1 levels were determined by Western blot of blood samples. Survival time was recorded, and data were analyzed using log-rank test.

Results
Assessment of cIAP1, Caspase-8, and SMAC Protein Levels in CD34+38− Stem/Progenitor Cells and CD34+ AML Cells
The expression of cIAP1, caspase-8, and SMAC in samples from 511 newly diagnosed AML patients and in CD34+ cells from 21 normal control subjects was measured (data not shown). Caspase-8 was statistically significantly higher in AML samples than normal control subject samples (P < .002), whereas no statistically significant difference was found in cIAP1 and SMAC levels. Expression levels were then measured in CD34+ and CD34+38− cells isolated from the blasts of 31 AML patients. Although no statistically significant differences between bulk and CD34+ cells were observed, the expression levels of cIAP1 and caspase-8 were statistically significantly higher in CD34+38− AML cells (mean = 1.40, SD = 0.44; mean = 2.59, SD = 1.83, respectively) than in bulk (mean = 0.95, SD = 0.24; mean = 1.00, SD = 0.28, respectively; P < .001) or CD34+ AML cells (mean = 1.04, SD = 0.26; mean = 1.00, SD = 0.31, respectively; P < .001 (Figure 1). The levels of SMAC were statistically significantly lower in CD34+38− AML cells than in bulk (mean = 0.53, SD = 0.33; mean = 1.23, SD = 0.39, respectively; P < .001) or CD34+ AML cells (mean = 1.26, SD = 0.40; P < .001) (Figure 1). This finding indicated a high cIAP1/SMAC ratio AML stem/progenitor cells and thus the potential for SMAC mimetics to target these cells.

Evaluation of IAPs and Caspase-8–Dependent Apoptosis in Birinapant-Treated AML Cells Cultured Under Various Conditions
Molm13 cells were cultured under various conditions and treated with birinapant (Figure 2A). Coculture increased, whereas hypoxia decreased, the expression of cIAP1, cIAP2, and XIAP (Figure 2B, lanes with arrows). Birinapant greatly decreased cIAP1 and, to a much lesser extent, cIAP2, and XIAP under various conditions (Figure 2B), suggesting that its activity is not diminished under coculture and hypoxic conditions. Similar results were shown in OCI-AML3 cells (Supplementary Figure 1A, available online).

Birinapant had limited activity against Molm13 cells. The combination of birinapant and tumor necrosis factor α (TNF-α or

Figure 1. Comparison of expression levels of total caspase-8, cellular inhibitor of apoptosis protein-1 (cIAP1), and second mitochondrial-derived activator of caspases (SMAC) in bulk acute myeloid leukemia (AML) blasts, CD34+ cells, and CD34+38− stem/progenitor cells isolated from blasts of 31 AML patients. Protein levels were determined by reverse-phase protein array. One-way analysis of variance, followed by Tukey’s multiple comparison test, was used to compare levels of proteins in bulk, CD34+, and CD34+38− cells. Error bars represent the standard deviation (SD) of the mean value for each group. All P values are two-sided.
TNF-related apoptosis-inducing ligand (TRAIL) was highly synergistic (i.e., CI < 0.01, CI < 0.001, CI = 0.05 ± 0.06, or CI = 0.05 ± 0.04 for birinapant plus TNF-α; CI < 0.01, CI < 0.01, CI = 0.07 ± 0.05, CI = 0.04 ± 0.03 for birinapant plus TRAIL) under standard, MSC co-culture, hypoxia, or MSC co-culture and hypoxia, respectively (Figure 2C), in agreement with the literature (13,17,18,35). Although slightly less effective under coculture than culture under standard conditions, the combination effect remained synergistic. The combinations were even more effective under hypoxia and coculture/hypoxia conditions (the P values of the combinations under different culture conditions are shown in Figure 2C), which was an unexpected finding. Similar results were observed using
OCI-AML3 cells (Supplementary Figure 1, available online), which conferred that the activation of the extrinsic pathway was a crucial mechanism of apoptosis induction. Although MSCs offered more protection to the attached (Supplementary Figure 1, B and C, lower panels) than the unattached OCI-AML3 cells (Supplementary Figure 1, B and C, upper panels), the combinations were able to synergistically induce apoptosis in both scenarios. Birinapant induced apoptosis in Jurkat but not in their caspase-8–deficient (36) counterparts (Supplementary Figure 2, available online), further supporting the activation of the extrinsic pathway as a main mechanism of cell death.

Examining Birinapant-Induced Apoptosis in Primary AML Cells, Including AML Stem/Progenitor Cells, and Normal CD34+ Cells

Cells from AML patients samples described in Table 1 were treated with birinapant. This agent induced apoptosis in bulk AML cells (Figure 3A), which was diminished when combined with an anti-TNF-α antibody (Supplementary Figure 3, available online). Notably, it also induced cell death in AML stem/progenitor cells with a similar efficacy (Figure 3B), and it effectively induced apoptosis in bulk and AML stem/progenitor cells cocultured with MSCs under hypoxia (Figure 3C). Furthermore, birinapant at concentrations effective against CD34+ AML cells were seemingly nontoxic to normal CD34+ cells cultured under various conditions (Figure 3D).

Appraisal of NFκB and Apoptotic Proteins in AML Cells and Apoptosis Induction by Birinapant, Demethylating Agents, and Their Combination

SMAC mimetics are known to target cIAPs, resulting in suppression of canonical NFκB signaling and stabilization of NFκB-inducing kinase (NIK), which leads to activation of noncanonical NFκB signaling (17) and increase in FLIP (37). Birinapant certainly suppressed canonical NFκB signaling as determined by decreased NFκBp65 and Bcl-xL and increased IκBα expression, and it activated noncanonical NFκB signaling as assessed by increased NIK, NFκBp52, and FLIP expression (Figure 4A). The activation of noncanonical NFκB signaling was further confirmed by increased nuclear translocation of p100/p52 subunits in the AML cells (Figure 4B).

Figure 3. Effects of birinapant (bir) on apoptosis induction in primary acute myeloid leukemia (AML) cells, including CD34−38− AML stem/progenitor cells, and normal CD34+ cells. A) Mononuclear cells from AML patients were treated with birinapant, and apoptosis was assessed at 24 and 48 hours. B) Mononuclear cells from AML patients were treated with birinapant, and apoptosis was assessed at 48 hours in bulk and CD34−38− cells. C and D) Mononuclear cells from AML patients and normal bone marrow (BM) control subjects were cultured under various conditions and treated with birinapant. Apoptosis was determined at 48 hours in bulk and CD34−38− cells from AML patients (C) and in CD34+ cells from AML patients and normal BM controls (D). The results are expressed as the mean ± standard deviation (error bars).
Figure 4. Expression of various proteins in OCI-AML3 cells treated with birinapant or demethylating agents. A and B) OCI-AML3 cells were treated with birinapant (bir) for 24 hours. Protein levels in total cell lysates (A) or cytoplasmic/nuclear fractions (B) were determined by western blot. C and D) OCI-AML3 cells were treated with decitabine (DAC) or 5-azacytidine (5-Aza) (C) or birinapant, DAC, 5-Aza, birinapant plus 5-DAC, or birinapant plus 5-Aza (D) for 48 hours and protein levels in total cell lysates were determined by western blot analysis.
To potentially enhance the therapeutic potency of birinapant and maximize activation of the extrinsic apoptosis pathway in AML, OCI-AML3 cells were cotreated with demethylating agents. Decitabine (DAC) or 5-Aza alone increased caspase-8 and XAF-1 (Figure 4C). Like birinapant, both agents also decreased levels of cIAP1, cIAP2, XIAP, and NFκB p65 and increased 1xBt. However, in contrast with birinapant, they both decreased the protein levels of FLIPL, NIK, and NFκBp100/p52. This effect was further enhanced by the birinapant and demethylating agent combination. Specifically, the combinations further decreased IAPs, NFκBp65, and NIK expression and suppressed birinapant-induced NFκBp52 and FLIPL expression (Figure 4D), suggesting that demethylating agents synergize with birinapant in AML cells. Molm13 cells were treated with birinapant, 5-Aza, or DAC; birinapant plus 5-Aza; or birinapant plus DAC. These combinations were highly synergistic under various culture conditions (Figure 5A), particularly under coculture and hypoxia. Similar results were obtained in OCI-AML3 cells (Supplementary Figure 4, available online).

Because birinapant most profoundly decreases levels of cIAP1, we postulated that cIAP1 reduction by birinapant likely contributed to 5-Aza synergy. OCI-AML3 cells were transfected with cIAP1 siRNAs (BIRC2_2, 3, 7, and 8) and treated with 5-Aza. All four siRNAs left-shifted the 5-Aza dose–response curves and decreased the protein levels of cIAP1 (Figure 5B), suggesting that the synergy of birinapant with 5-Aza was mediated, at least in part, through birinapant-induced cIAP1 reduction.

Remarkably, birinapant and 5-Aza or DAC were highly synergistic not only in bulk (Figure 5C; Supplementary Figure 5A, available online; n = 14) but also in CD34+38 AML cells (Figure 5D; Supplementary Figure 5B, available online; n = 10) (summarized in Table 1) cocultured with MSCs under hypoxia. This finding suggested that these drug combinations were highly effective under coculture/hypoxic conditions that mimic the BM microenvironment. Interestingly, various birinapant combinations in the NCI-60 cell lines revealed that the combination of birinapant and 5-Aza demonstrated the strongest additive/synergistic effects on not only leukemia but also other cancer cell types (ie, 37 of 60 cancer cell types) (Supplementary Figure 6, available online). These results further suggest a more generalizable synergistic effect, beyond what we have shown in malignant hematopoietic cells, as a cancer therapeutic strategy.

Assessment of Birinapant Alone or Combined With 5-Aza in a Xenograft Mouse Model of Human AML
Molm13-GFP/Luc cells were injected into mice (n = 10 per group) and drug treatment was initiated on day 3 after inoculation (Figure 6). On day 11, an antileukemia effect of birinapant and other agents was observed in all treatment groups. Control mice showed high leukemia burden on day 17. Birinapant alone decreased this tumor burden, and the combination of birinapant and 5-Aza was most effective in this regard (Figure 6A). Relative intensities determined by image, expressed as mean ± SD, were 7.24 ± 0.42, 7.73 ± 0.37, 8.85 ± 0.11, or 9.28 ± 0.34 for birinapant plus 5-Aza, 5-Aza, birinapant, or control, respectively (P = .05 for combination vs 5-Aza; P < .001 for combination vs birinapant or control). Birinapant also decreased, which was further enhanced by combination with 5-Aza, the number of peripheral blood leukemic cells at day 19 (Figure 6B). Viable human CD45+ cell counts, expressed as mean ± SD, were 0.22 ± 0.08, 0.35 ± 0.09, 1.91 ± 0.70, or 4.59 ± 0.17 for birinapant plus 5-Aza, 5-Aza, birinapant, or control, respectively (P = .02 for combination vs 5-Aza; P = .01 for combination vs birinapant; P < .001 for combination vs control). Moreover, birinapant induced a rapid and sustained decrease of cIAP1 levels (Figure 6C). After being killed for tissue collections, 7 or 8 mice per group were used to determine median survival time. The mice treated with 5-Aza or birinapant had statistically significantly longer median survival time (26 days, P < .001; 22 days, P < .001, respectively) than control mice (18 days), and the mice treated with birinapant plus 5-Aza had a statistically significantly longer median survival time (30 days) than mice treated with either 5-Aza (P = .04 vs 5-Aza) or birinapant alone (P < .001 vs birinapant) (Figure 6D).

Discussion
The SMAC mimic birinapant promotes death receptors/caspase-8–mediated extrinsic apoptosis, and as a single agent, birinapant was active against primary AML cells. However, its antileukemia activity was markedly diminished when birinapant was combined with an anti-TNF-α antibody, suggesting that primary AML cells produce TNF-α. Here, we demonstrated that combinations of birinapant and demethylating agents acted against AML synergistically in vitro and more effectively than either agent alone in vivo. Demethylating agents induce DNA hypomethylation, which results in the increased expression of tumor suppressor genes that are silenced in cancer cells (38,39). However, the mechanisms of action of these agents in AML are not entirely clear (40). Both DAC and 5-Aza increase the expression of genes that lack promoter CpG islands in AML cells (41) and inhibit NFκB activity (42). In this study, demethylating agents affected several proteins in the extrinsic apoptosis pathway (Figure 7). Epigenetic silencing of caspase-8 (43–46) and XAF-1 (47,48) have been reported in various malignant cell types. 5-Aza and DAC increased the expression of caspase-8 and XAF-1 in AML cells (Figure 7, green arrows), which is consistent with their proposed demethylating activity.

IAPs are regulated by NFκB (49–51) and IAPs; in particular, cIAPs are known to positively regulate canonical and negatively regulate noncanonical NFκB pathways (52). Consistent with these findings, birinapant suppressed the canonical NFκB pathway by decreasing IAP expression but stabilized NIK, apparently activating the noncanonical NFκB pathway. Birinapant also increased FLIPL expression, which is in agreement with a report demonstrating that NIK negatively regulates SMAC mimetic-induced apoptosis by inducing FLIP production (37). DAC and 5-Aza not only inhibited the canonical NFκB pathway and decreased IAP expressions that enhanced birinapant activity, but they also inhibited the noncanonical NFκB pathway and decreased FLIPL levels (Figure 7, blue arrows).

The ability of demethylating agents to increase proapoptotic caspase-8 and XAF-1, and decrease antiapoptotic IAPs and FLIPL, all of which directly or indirectly regulate the extrinsic apoptosis pathway, likely contributed to their cytotoxic synergy when combined with birinapant. Birinapant’s ability to decrease IAP proteins, in particular cIAP1, efficiently under various culture conditions also likely contributed to its synergy with demethylating agents. Recent reports have...
Figure 5. Effects of combinations of birinapant (bir) and demethylating agents on apoptosis induction in acute myeloid leukemia (AML) cells. A) Molm13 cells were cultured under various conditions and treated with birinapant, 5-azacytidine (5-Aza), birinapant plus 5-Aza or birinapant, decitabine (DAC), or birinapant plus DAC. Cell death was determined at 72 hours by annexin V staining in the presence of 7-aminoactinomycin D. B) OCI-AML3 cells were transfected with cellular inhibitor of apoptosis protein-1 (cIAP1), small interfering RNAs (siRNAs) and treated with 5-Aza for 96 hours (left panel) or transfected with siRNAs for 48 hours (right panel). Cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay. Western blot analysis was used to determine cIAP1 levels. Buffer indicates treatment with siRNA buffer and transfection reagent only, NS indicates treatment with a negative control siRNA with no known targets, and BIRC2_2, _3, _7, and _8 represent four different sequences targeting cIAP1. C and D) Mononuclear cells from AML patients were cultured under various conditions and treated with birinapant, 5-Aza, or birinapant plus 5-Aza for 48 hours. Cell death was determined in bulk (n = 14) (C) and CD34⁺38⁻ cells (n = 10) (D). Combination index (CI) values under various culture conditions are shown on the top of the graphs. CI values were determined using CalcuSyn software and expressed as the mean ± standard deviation (error bars) of the CI values obtained at the effective dose for 50, 75, and 90% of the treatment population (i.e., ED₅₀, ED₇₅, and ED₉₀, respectively). Two-sided P values were calculated by a paired Student t test.
Figure 6. Antileukemia activity of birinapant (bir) and birinapant plus 5-azacytidine (5-Aza), in a Molm13 NOD/SCID IL2Rγnull (NSG) mouse model. Birinapant and birinapant plus 5-Aza decreased luciferase activity (A) and circulating Molm13 cells (B). Numbers given are the means, and the error bars represent the standard deviations. Two-sided P-values were calculated using a paired Student t test. Birinapant induced a rapid and sustained decrease in the cellular inhibitor of apoptosis protein-1 (cIAP1) in the blood samples (collected immediately before, or 5 h after, treatment) from the Molm13 NSG mice (C). D) Birinapant alone increased survival of the Molm13 NSG mice, which was further enhanced by co-treatment with 5-Aza. The number of mice at risk at different time points in the different groups is given below the survival curves in (D). Neg CON = negative control.
suggested cIAP2 is a resistance factor for SMAC mimetics (53,54), which we did not observe in the AML cells examined. Combinations of SMAC mimetics and demethylating agents that decrease all IAP proteins likely overcome this resistance. Importantly, IAP and NFκB pathway proteins were further decreased when birinapant was combined with demethylating agents. In the AML mouse model, whereas birinapant combined with 5-Aza was statistically significantly more effective, 5-Aza alone at 5 mg/kg was highly active, suggesting that higher synergism can be achieved by lowering doses of 5-Aza.

The finding that AML stem/progenitor cells express high levels of cIAP and low levels of SMAC may partially explain why they are more resistant to apoptosis inducers than bulk AML cells, which strongly supports the use of SMAC mimetics to eliminate CD34+38- AML cells. The increased expression of caspase-8 may offset the high expression of cIAP1 in AML stem/progenitor cells, making them as sensitive as bulk cells to birinapant. This is supported by the effectiveness of birinapant against bulk as well as CD34+38- AML cells. Importantly, birinapant was effective against AML cells, including AML stem/progenitor cells cultured with MSCs under hypoxia that mimicked in vivo conditions in patient (22,25), suggesting that birinapant can overcome the protection of leukemia cells provided by the BM microenvironment. Importantly, birinapant had negligible toxicity against normal CD34+ cells at conditions that were toxic to AML CD34+ cells. This finding agrees with reports by others using different SMAC mimetics in cytotoxicity (19) or using colony assays (55). Interestingly, although no statistically significant difference in cIAP1 and SMAC between AML blasts and normal controls, higher total caspase-8 (the effector caspase for SMAC mimic-mediated apoptosis) in AML blasts may suggest that AML cells are more susceptible to cIAP inhibition and thereby more sensitive to birinapant than normal controls. Finally, the additive/synergistic effects of the birinapant and 5-Aza combination in AML, as well as many NCI-60 cell lines from different tumor types, suggests a potential broad application of this treatment strategy in cancer therapy.

Our study is not without limitations. Although we tried to examine the effects of birinapant and its combinations with demethylating agents on AML bulk and stem/progenitors cells under conditions that mimic the physiological BM microenvironment (cocultures with MSC and under hypoxia), in vitro and in vivo, in a murine model of human xenographed leukemia, these conditions are not the same as in humans. The therapeutic potential of the combination can only be evaluated in clinical trials, which are currently ongoing. Furthermore, a comprehensive gene expression analysis is needed to further the understanding of the mechanism underlying the observed striking synergy of birinapant and demethylating agents.

References


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