Targeting AML stem/progenitor cells by combinational therapy with SMAC mimetics and demethylating agents

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Chemotherapy for acute myeloid leukemia (AML) principally induces intrinsic apoptosis in circulating leukemic blasts. Unfortunately, standard therapy is relatively ineffective in eliminating AML stem/progenitor cells that drive leukemogenesis. The inhibitors of apoptosis (IAP) protein family are critical regulators of cell survival. IAP proteins, in particular cIAP1 and cIAP2, have drawn great attention in recent years as targets for cancer therapy due to the development of SMAC mimetics. We discovered that the expressions of cIAP1, which primarily inhibits the extrinsic apoptosis pathway and a main target of SMAC mimetics, and caspase-8, the key caspase of the extrinsic apoptosis were significantly higher in AML stem/progenitor cells than in bulk AML cells. Conversely, the expression of SMAC, an endogenous antagonist of IAP proteins, was significantly lower by comparison. We investigated the therapeutic potential of targeting IAPs by SMAC mimetics in AML and reported that the novel SMAC mimetic birinapant effectively induced apoptosis in AML cells, including AML stem/progenitor cells. This effect was present even in leukemic cells co-cultured with bone marrow derived-mesenchymal stromal cells under hypoxic conditions representative of the bone marrow microenvironment. Furthermore, this anti-leukemia activity was enhanced in vitro and in vivo by combination with demethylating agents, which apparently modulated NFκB signaling and many key members of the extrinsic apoptosis pathway. The apoptosis repressor with caspase recruitment domain (ARC) protein is known to suppress the extrinsic apoptosis. We have demonstrated that ARC is an effective negative prognostic factor for AML, is regulated by cIAP1/NIK signaling in AML cells, and can be inhibited by demethylating agents. Our findings suggest that the activation of the extrinsic pathway has the potential to eradicate AML stem/progenitor cells and that mechanistic combinations of SMAC mimetics with agents that modulate the extrinsic apoptosis pathway may benefit patients with AML. In fact, our preliminary results served as the catalyst for recently-initiated phase I/II trials of birinapant in combination with 5-azacytidine in pre-leukemia myelodysplastic syndrome and AML patients.

Keywords: AML; IAP proteins; SMAC mimetics; demethylating agents; apoptosis; ARC; NFκB

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Despite recent treatment improvements, acute myeloid leukemia (AML) remains a disease with a poor clinical outcome. Although most patients respond to chemotherapy initially, many ultimately relapse and succumb to their disease. Evasion and/or development of resistance to standard chemotherapeutic agents, especially by AML stem/progenitor cells (LSC), is a leading cause of disease relapse and a major obstacle to clinical success in the treatment of this disease.
Apoptosis is genetically controlled by multiple regulators, frequently deregulated in malignant cells, and is thus one of the hallmarks of cancer [1]. Indeed, resistance to cell death contributes not only to tumor initiation and disease progression, but also drug resistance. Consequently, anti-apoptotic proteins are potentially excellent targets for cancer therapy. Inhibitors of apoptosis (IAP) proteins are a family of evolutionarily-conserved proteins that are important modulators of the apoptosis machinery. They have one to three copies of a well-conserved domain of ~70 amino-acid known as baculovirus IAP repeat (BIR). Currently, eight members of IAP proteins have been described in human cells. They directly, or indirectly, regulate caspase activity and cell survival though their BIR domains and/or ubiquitin-ligase RING zinc finger activity. IAPs are frequently aberrantly expressed in human cancers due to genetic aberrations; inappropriate induction by growth factors, cytokines, oncogenes, or multiple signaling pathways that are often constitutively active in malignant cells, and their microenvironments; or the loss of tumor suppressors or endogenous IAP inhibitors. The pro-survival function of IAP proteins, their overexpression in leukemic cells, and their roles in treatment resistance make them attractive therapeutic targets for cancer therapy, including AML.

Survivin is the smallest IAP protein containing only one BIR domain [2], and it is the only IAP protein that plays roles in both cell cycle regulation and the inhibition of cell death. Overexpression of survivin has been observed in a number of hematological malignancies including AML [3]. We recently profiled survivin expression in samples from 511 newly diagnosed AML patients using a reverse-phase protein array method followed by multivariate Cox model analysis and found that a higher survivin level predicted shorter overall survival /event-free survival [4]. We also discovered that more primitive CD34^+38^- LSC had correspondingly higher survivin levels than bulk AML cells [4], suggesting this protein has a role in LSC survival, self-renewal, and disease relapse. Thus survivin is an attractive target for AML therapy. Indeed, survivin inhibition by antisense oligonucleotides (ASOs) induced cell proliferation defects and subsequent cell death in AML cells [5].

XIAP is the most potent IAP protein that suppresses both the intrinsic and extrinsic apoptosis pathways. XIAP is highly expressed in AML and upregulated by cytokines and the MAPK/PI3K signaling pathway, which supports its pro-survival role in leukemia [6]. The prognostic impact of XIAP expression in AML remains debatable [7-9]. Nevertheless, inhibition of XIAP by ASO induces apoptosis in, and enhances the chemosensitivity of, leukemic cells [9]. The inhibition of XIAP activity by small-molecule XIAP inhibitors promoted apoptotic cell death in both leukemic cell lines and primary AML blasts [10].

Furthermore, XIAP is a downstream effector of multiple signal transduction cascades. It also activates NFκB [11, 12] and PI3K/AKT [13] signaling, which suggests that inhibition of XIAP has potentially broad therapeutic implications. Interestingly, in a phase 1/2 study of XIAP ASO in patients with relapsed/refractory AML, we demonstrated that XIAP inhibition induced apoptosis preferentially in CD34^+38^- LSC, which was associated with a clinical response [14].

Although most early studies focused on survivin and XIAP, cIAP1 and cIAP2 have attracted great attentions in recent years because of the critical roles for these proteins play in cell survival and NFκB signal transduction, hence the development of a new class of anticancer agents, known as SMAC mimetics, to block their activity. SMAC is a mitochondrial protein and an endogenous inhibitor of IAP proteins. Once matured in the cytoplasm, SMAC binds to IAP proteins predominantly via its N-terminal tetrapeptide AVPL sequence, suppresses XIAP-mediated caspase inhibition, and causes cIAP auto-ubiquitination and degradation leading to apoptosis [15]. Little, or no, expression of SMAC has been reported in various solid tumors, which also correlated with poor treatment outcomes [16-18]. Currently several SMAC mimetics, which mimic the N-terminal tetrapeptide AVPL sequence, have entered clinical trials in solid tumors as well as hematological malignancies either as a single agent or in combinational therapy with other chemotherapeutic drugs.

We treated cells from AML cell lines with birinapant, a potent and perhaps the most clinically-advanced SMAC mimetic, and demonstrated that this agent potently decreased cIAP1, and to a much less degree of cIAP2 and XIAP, in AML cells, even when these cells were co-cultured with bone marrow derived mesenchymal stromal cells (MSCs) under hypoxia (1% O2), a condition mimicking the bone marrow microenvironment, and induced the extrinsic apoptosis [19].

The activation of extrinsic apoptosis is initiated by death receptor-ligand binding, which transduces intracellular death signaling mediated by caspase-8 activation [20]. This pathway can be blocked by various inhibitors such as cIAPs, FLIP [21], and the apoptosis repressor with caspase recruitment domain (ARC) protein [22, 23] (Figure 1). Birinapant mimics SMAC to target cIAP1 and thus induces caspase-8 mediated extrinsic apoptotic cell death. Before assessing birinapant’s therapeutic potential in AML, we determined the expression of SMAC, cIAP1, and caspase-8 in a large cohort of newly diagnosed AML patient samples (n = 511) by reverse-phase protein array. We found that all three proteins are variably
expressed in AML samples, but interestingly, the expression of cIAP1 and caspase-8 was significantly higher, and SMAC significantly lower, in CD34+38- LSC than CD34+ and bulk AML cells [19], suggesting that SMAC mimetics, such as birinapant, have the potential to target AML cells, including LSC. Indeed, birinapant induced a time- and dose-dependent cell death in cells from patients with AML; doing so in bulk as well as CD34+38- cells. This effect was not attenuated even when these cells were co-cultured with MSCs under hypoxic conditions. Importantly, while very active against CD34+ AML cells, birinapant had minimal toxicity in normal CD34+ hematopoietic cells [19].

To increase its therapeutic potential, we combined birinapant with various nucleotide analogues currently in clinical use, or under development, for AML. We found that combinations of birinapant with cytarabine, clofarabine, 5-azacytidine (5-Aza), or dacitabine (DAC) all induced death of AML cells in a synergistic manner. This synergy was particularly strong when birinapant is combined with demethylating agents [19, 24].

The suppression of gene expression through epigenetic DNA modification is a mechanism through which cancer cells block the expression of tumor suppressor and related anti-cancer genes. Hence, this is the rationale for therapies using demethylating agents such as 5-Aza and DAC in patients with AML and pre-leukemic myeloid dysplastic syndrome (MDS). In the context of the extrinsic apoptosis cascade, it was reported that caspase-8 [25-28] and XAF-1, a XIAP antagonist protein [29, 30] are methylated in many malignant cells. We treated OCI-AML3 cells with 5-Aza or DAC and found indeed increased expression of both proteins. However, the increases were minimal and therefore could not explain the highly synergistic activity of the drug combination.

In addition to a direct role in apoptosis regulation, IAPs, particularly cIAPs also have a role in NFκB signaling. cIAP1 can activate canonical NFκB signaling, while inhibiting the non-canonical pathway by inducing the proteasomal degradation of the NFκB inducing kinase (NIK), a kinase required for activation of non-canonical NFκB [31]. SMAC mimetics degrade cIAP1 leading to the induction of non-canonical NFκB signaling. Indeed, treating AML cells with birinapant increased NIK, activated non-canonical NFκB [31].

The overexpression of ARC in AML cells diminished birinapant-induced apoptosis, while ARC suppression sensitized these cells to birinapant-induced cell death. Furthermore, the inhibition of ARC in MSCs rendered these cells less protective of AML cells against birinapant-induced cell death [35].
An increase in the expression of caspase-8 and XAF-1 was consistent with the mechanism of action of 5-Aza and DAC, since these agents trigger the re-expression of tumor suppressor genes that are silenced in cancer cells [36, 37]. However, the mechanisms of action of these agents in AML are not entirely clear [38]. Reports have shown that DAC and 5-Aza increase the expression of genes that lack promoter CpG islands in AML cells [39] and inhibit NFκB activity [40].

To further understand the mechanism of synergy, we determined that the expression of IAPs and components of NFκB signaling in DAC- and 5-Aza-treated AML cells and found that demethylating agents decrease XIAP, cIAP1, cIAP2 and both canonical and non-canonical NFκB signaling. Combinations of birinapant with 5-Aza or DAC further decrease IAP proteins and canonical NFκB signaling, and suppressed birinapant-induced non-canonical NFκB signaling [19]. As expected, treatment of AML cells with demethylating agents decrease the levels of FLIP and ARC [19, 35], which are regulated by NIK and provide resistance against caspase-8-mediated apoptosis. Figure 2 summarizes the putative mechanisms of the synergy of combination of birinapant and demethylating agents.

To determine a potential therapeutic relevance of this combination in AML therapy, we treated AML patient samples with birinapant, 5-Aza, or DAC alone and with the birinapant demethylating agent combinations under various co-culture conditions and found that the combination treatments were significantly more effective than the single agents in killing AML cells, including the CD34+38- LSC, even when these cells were co-cultured with MSCs under hypoxic conditions. We next treated human AML cell-xenografted NOD/SCID IL2Rnull mice with birinapant, 5-Aza, and the birinapant-5-Aza combination. Birinapant treatment alone was able to decrease leukemia burden in mice and prolong their survival. However, this was significantly improved when birinapant was combined with 5-Aza [19].

Cells from the NCI-60 panel of cell lines were treated with birinapant in combination with various therapeutic agents including 5-Aza, lapatinib, vorinostat, dacarbazine, and melphalan. Interestingly, the most profound additive/synergistic effects were observed with birinapant and its combination with 5-Aza not only in the AML cells tested, but also in cells representing many different tumor types included in the NCI-60 panel [19]. This suggested a potentially broad application for this treatment strategy in cancer therapy. We are currently investigating targeting other cell survival pathways to sensitize with the activity of SMAC mimetics.

Collectively, our findings suggest that the activation of the extrinsic pathway has great potential to eradicate LSC, which are often resistant to current therapy, and that combinational therapy with SMAC mimetics and other therapeutic agents that modulate the extrinsic apoptosis may have added benefit in the therapy of patients with AML. Based on our findings, a phase Ib/Ia study of birinapant in combination with 5-Aza in patients with MDS who are naïve to, refractory to, or have relapsed on 5-Aza is currently ongoing.

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