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Smac mimetic primes apoptosis-resistant acute myeloid leukaemia cells for cytarabine-induced cell death by triggering necroptosis

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ABSTRACT

The prognosis for patients with acute myeloid leukaemia (AML) is still poor, thus calling for novel treatment strategies. Here, we report that the small-molecule Smac mimetic BV6, which antagonizes Inhibitor of Apoptosis (IAP) proteins, acts in concert with cytarabine (AraC) to trigger cell death in AML cells in a highly synergistic manner (combination index 0.02–0.27). Similarly, BV6 cooperates with AraC to trigger cell death in primary AML samples, underscoring the clinical relevance of our findings. Molecular studies reveal that the TNFα-blocking antibody Enbrel significantly reduces BV6/AraC-induced cell death, demonstrating that an autocrine/paracrine TNFα loop mediates cell death. Furthermore, BV6 and AraC synergize to induce loss of mitochondrial membrane potential, caspase activation and DNA fragmentation, consistent with apoptotic cell death. Nevertheless, the caspase inhibitor zVAD.fmk fails to protect against BV6/AraC-induced cell death. Intriguingly, this cell death upon caspase inhibition is significantly reduced by pharmacological inhibition of two key components of necroptosis signaling, i.e. by RIP1 kinase inhibitor Necrostatin-1 or MLKL inhibitor NSA. Thus, BV6 sensitizes AML cells to AraC-induced cell death and overcomes apoptosis resistance by triggering necroptosis as alternative form of cell death. These findings have important implications for Smac mimetic-based strategies to bypass apoptosis resistance of AML.

1. Introduction

Acute myeloid leukaemia (AML) represents the most frequent type of myeloid malignancy in adulthood [1]. Programmed cell death by apoptosis plays a pivotal role in maintaining tissue homeostasis in the lympho-haematopoeitic system, as a tight balance between cell death and proliferation is necessary to keep the fast turnover of cells in this compartment in check [2,3]. Too little cell death not only facilitates leukaemogenesis, but also promotes treatment resistance, since most antileukemic therapies act by triggering cell death [2,3].

Apoptosis represents one of the best characterized forms of programmed cell death [4] and typically proceeds via two key signal transduction pathways [5]. In the extrinsic (death receptor) pathway, ligand-mediated engagement of death receptors such as tumour necrosis factor (TNF) receptor 1 (TNFR1) or CD95 triggers activation of caspase-8 and subsequently of effector caspases such as caspase-3 [6]. In the intrinsic (mitochondrial) pathway, the release of mitochondrial intermembrane space proteins such as cytochrome c into the cytosol leads to caspase-3 activation, while second mitochondria-derived activator of caspases (Smac) promotes apoptosis by neutralizing "Inhibitor of apoptosis" (IAP) proteins [7].

Besides apoptosis, there are additional modes of programmed cell death [4]. For example, necroptosis represents a recently identified form of programmed necrosis that typically occurs under circumstances in which caspase activation is absent or blocked [8]. The serine/threonine kinase Receptor-Interacting Protein 1 (RIP1) represents a key regulator of necroptosis that phosphorylates RIP3 to form the necrosome complex [8], which in turn phosphorylates mixed lineage kinase domain-like protein (MLKL) that has recently been identified as a new component of the necroptosis signaling pathway [9]. Apoptotic and necroptotic pathways are closely interconnected, e.g. by caspase-8 that cleaves RIP1, thereby shutting down necroptosis [10].

Abbreviations: AML, acute myeloid leukaemia; AraC, Cytarabine; BIR, Baculovirus IAP Repeat; CBF, core-binding factor; CI, combination index; cIAP1, cellular inhibitor of apoptosis protein 1; FACS, fluorescence-activated cell-sorting; FADD, FAS-associated death domain protein; IAP, Inhibitor of apoptosis; MLKL, mixed lineage kinase domain-like protein; MMP, mitochondrial membrane potential; Nec, Necrostatin-1; NF-κB, Nuclear Factor kappaB; PI, propidium iodide; RING, Really Interesting New Gene; RIP1, Receptor-Interacting Protein 1; Smac, second mitochondria-derived activator of caspases; TNF, tumour necrosis factor; TNFR1, tumour necrosis factor receptor 1; XIAP, X-linked inhibitor of apoptosis; zVAD.fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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Programmed cell death is typically impaired in cancer cells, for example by the dominance of mechanisms that prevent the induction or execution of cell death [11]. For example, X-linked inhibitor of apoptosis (XIAP), one of the eight human IAP proteins, has been demonstrated to inhibit caspase-9, -7 and -3 via its Baculovirus IAP Repeat (BIR) domains [12], while cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2 predominately function as E3 ubiquitin ligases via their Really Interesting New Gene (RING) domain [13,14]. K63-linked ubiquitination of RIP1 by cIAP1/cIAP2 promotes activation of the canonical Nuclear Factor kappaB (NF-κB) signaling cascade [13], while deubiquitinated RIP1 facilitates programmed cell death. Depending on the cellular context, RIP1 forms a complex with FAS-associated death domain protein (FADD) and caspase-8 to trigger apoptosis or, alternatively, engages necroptosis via the necrosome, a complex of RIP1 with FADD and RIP3 [8].

Aberrantly high expression levels of IAP proteins have been detected in various human malignancies and have been linked to poor prognosis [15]. In AML, high expression of XIAP protein has been shown to correlate with adverse outcome [16,17] and high cIAP2 mRNA expression as part of a three-gene expression signature was associated with reduced survival [18]. Furthermore, deregulated apoptotic signaling and high IAP protein levels have recently been identified in a clinically distinct subgroup of core-binding factor (CBF) AML with poor clinical outcome [19,20].

Against this background, small-molecule inhibitors against IAP proteins including Smac mimetic have been developed in recent years to reactivate cell death pathways in cancer cells [15]. Smac mimetic has been shown to interfere with the XIAP-imposed inhibition of caspases, to trigger autoubiquitination and proteasomal degradation of cIAP proteins [21–23] and to reduce the ubiquitination of RIP1 [13]. Clinical trials testing Smac mimetics as single agents and in combination therapies have been launched in solid cancers and lymphoma [15]. Recently, a phase I/II clinical trial that evaluates monotherapy with the Smac mimetic TL32711 has been initiated in relapsed elderly AML patients (www.clinicaltrials.gov).

Our preclinical studies in various cancer entities demonstrated that Smac mimetic can prime cancer cells for apoptosis in response to different cytotoxic stimuli such as death receptor ligands, chemotherapeutics or γ-irradiation [24–31]. Searching for novel drug combinations to enhance the efficacy of antileukemic therapies, in the present study we investigated the question whether or not the small-molecule Smac mimetic BV6 can increase chemosensitivity of AML cells towards AraC, a key chemotherapeutic drug used in the treatment of AML [32].

2. Materials and methods

2.1. Cell culture

Human AML cell lines were obtained from ATCC (CEM, Manassas, VA, USA) or DSMZ (Braunschweig, Germany) and were cultured in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany), supplemented with 10% FCS (fetal calf serum) (Biochrom, Berlin, Germany), 1 mM glutamine (Invitrogen, Karlsruhe, Germany) and 1% penicillin/streptomycin (Invitrogen). BV6, a bivalent Smac mimetic that mimics the N-terminal part of the endogenous Smac protein and neutralizes XIAP, cIAP1 and cIAP2 [21], was kindly provided by Genentech, Inc. (South San Francisco, CA, USA). Caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was obtained from Bachem (Heidelberg, Germany), TNFα and Necrostatin-1 (Nec-1) from Biomol (Hamburg, Germany), AraC from Sigma (Steinheim, Germany), NSA from Toronto Research Chemicals Inc. (North York, CA). Enbrel was kindly provided by Pfizer. All chemicals were purchased by Sigma unless indicated otherwise. Bone marrow samples from AML patients were obtained at diagnosis before onset of therapy after informed consent and with the approval of the Ethics Committee of the University Hospital Frankfurt. Baseline morphology, cytogenetics and cell surface antigen analysis were performed as part of the routine diagnostical assessment. Diagnosis and classification of the AML were based on the criteria of the French-American-British (FAB) group. Characteristics of patients are summarized in supplemental Table 3. Mononuclear cells were isolated using Ficoll Isopaque (Amersham Bioscience, Freiburg, Germany). Cells were used for analysis either directly after Ficoll separation or after cryopreservation and were cultured in RPMI 1640 medium supplemented with 20% FCS, 1 mM glutamine, 1% penicillin/streptomycin, 4 μg/l NaSelen, 6 mg/l Insulin, 3 mg/l Transferin (1:167 ITS) (Invitrogen), 1 mM sodium pyruvate (Sigma) and 50 μM α-Thioglycerol (Sigma). Samples with spontaneous apoptosis ⩾40% at the time point of measurement were excluded from the analysis.

2.2. Western blot analysis

Western blot analysis was performed as described previously [33] using the following antibodies: mouse anti-caspase-8 from Alexis Biochemicals (Epalinges, Switzerland), rabbit anti-caspase-3 and rabbit anti-caspase-9 (Cell Signaling, Beverly, MA), mouse anti-XIAP from BD Biosciences (Heidelberg, Germany), goat anti-cIAP1 from R&D Systems, Inc. (Wiesbaden, Germany). Mouse anti-GAPDH (HyTest, Turku, Finland) or mouse anti-β-actin (Sigma) were used as loading controls. Goat-anti-mouse IgG, goat-anti-rabbit IgG or donkey-anti-goat IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies. Enhanced chemiluminescence was used for detection (Amersham Bioscience). All Western blots shown are representative of at least three independent experiments.

2.3. Determination of cell death

Apoptosis was determined by fluorescence-activated cell-sorting (FACS) analysis (FACScan, BD Biosciences) of DNA fragmentation of propidium iodide (PI)-stained nuclei as described previously [33]. Cell death was assessed by forward/side scatter analysis and flow cytometry as described previously [24] or by Annexin-V/PI staining (Roche) according to the manufacturer's instructions.

2.4. Determination of mitochondrial membrane potential

To determine the mitochondrial membrane potential, cells were incubated with TMRM (50 nM; Invitrogen) for 10 min at 37 °C and immediately analysed by flow cytometry.

2.5. Statistical analysis

Statistical significance was assessed by Student's *t*-test (two-tailed distribution, two-sample, unequal variance). Drug interactions were analysed by the combination index (CI) method based on that described by Chou [34] using CalcuSyn software (Biosoft, Cambridge, UK). CI < 0.9 indicates synergism, 0.9-1.1 additivity and >1.1 antagonism.

3. Results

3.1. Smac mimetic sensitizes AML cells to AraC-induced cell death

To investigate the question whether the small-molecule Smac mimetic BV6, which neutralizes XIAP, cIAP1 and cIAP2 [21], enhances the sensitivity of AML cells towards AraC, the key chemotherapeutic agent for the treatment of AML, we used several AML cell lines with different molecular characteristics (suppl. Table 1, suppl. Fig. 1). Importantly, BV6 acted in concert with AraC to induce cell death compared to treatment with AraC alone (Fig. 1A). Notably, this drug interaction between BV6 and AraC was highly synergistic as demonstrated by calculation of CI (0.02–0.27, suppl. Table 2). Kinetic analysis showed that BV6 increased AraC-mediated cell death in a time-dependent manner (Fig. 1B). To validate these results obtained in AML cell lines also in primary AML samples, we extended our experiments to freshly isolated leukemic blasts derived from patients with AML before the onset of chemotherapy. Patients' characteristics are summarized in suppl. Table 3. Importantly, BV6 synergized with AraC to induce cell death in 7 of 11 primary AML samples (Fig. 2A); as examples, quantitative data are shown for two patients (Fig. 2B). Together, this set of experiments demonstrates that the Smac mimetic BV6 synergistically acts in concert with AraC to trigger cell death in AML cells, including primary AML blasts. In the following, we explored the underlying molecular mechanisms responsible for the synergistic interaction of BV6 and AraC in AML cells.