Activation of necroptosis to overcome drug resistance in leukemia

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Received: September 13, 2016
Published online: October 24, 2016

The understanding of cell death mechanisms is crucial for the development and application of novel anti-cancer therapies to avoid or circumvent drug-resistance in refractory malignancies. Impairment of apoptotic cell death plays a major role in therapy resistance and relapse of acute lymphoblastic leukemia (ALL) patients. Therefore, efforts are being directed at new agents reactivating apoptosis or inducing alternative cell death pathways such as necroptosis, a regulated form of necrosis. In a recent study published in Science Translational Medicine we show that the IAP (inhibitor of apoptosis proteins) inhibitor birinapant potently induces cell death in patient-derived ALL cells in vitro and in vivo through a receptor-interacting protein kinase 1- (RIP1) dependent mechanism. To define the cell death modality induced downstream of RIP1, we used a multicolor lentiCRISPR approach that allows simultaneous knockout of multiple genes. We observed that apoptosis and necroptosis are induced simultaneously as the inhibition of both pathways is required to restore cell viability upon birinapant treatment. This induction of dual cell death makes birinapant and other IAP inhibitors interesting agents for the treatment of refractory or drug resistant malignancies.

Keywords: Necroptosis; drug resistance; receptor-interacting protein kinase 1; acute lymphoblastic leukemia


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Eliminating cancer cells is the ultimate goal of standard chemotherapies, radiotherapy, novel targeted therapies and in general all anticancer treatments. Most of those therapies rely on the induction of regulated cell death. Therefore, the understanding of programmed cell death pathways and the mechanisms by which they are induced and regulated is key for the development of such treatments.

Apoptosis is the best described pathway of programmed cell death. It is morphologically characterized by a decrease of the cellular volume, mitochondrial outer membrane permeabilization (MOMP), chromatin condensation, nuclear fragmentation as well as membrane blebbing. It can be classified as intrinsic or extrinsic depending on the signaling cascade mediating it. Intrinsic apoptosis can be induced by various intracellular stress signals, and it is mediated by the disruption of the balance of pro- and anti-apoptotic BCL2 family proteins. This leads to MOMP, the formation of a caspase-9 complex (termed apoptosome) and subsequent caspase-3 activation. On the other hand, extrinsic apoptosis is induced by the activation of death receptors by their extracellular ligands. This leads then to the recruitment of an intracellular death-inducing signaling complex (DISC) which activates the initiator caspases -8 and -10, which subsequently activate the effector caspases -3 and -7. The molecular mechanisms of apoptosis have been reviewed
Evasion of apoptosis is one of the hallmarks of cancer and is tightly linked to drug resistance, therapy failure and relapse. Apoptosis resistance is multifactorial, hence various mechanisms have been described by which cancer cells can escape apoptotic cell death. Some relevant escape mechanisms include de-regulation of the pro- and anti-apoptotic BCL2 family proteins, compensatory activation of autophagy (a regulated process of degradation and recycling of cellular components), and defects on death receptor signaling \[14-6\]. The inability of malignant cells to undergo apoptosis represents a major clinical challenge for the treatment of childhood acute lymphoblastic leukemia (ALL) among many other malignancies. Despite the increase in the long-term survival achieved in the last decades, around 20% of ALL patients show a poor clinical response to the frontline treatments and suffer relapse. Relapsed patients experience a dismal outcome. Because most anticancer drugs, including the frontline agents used for the treatment of ALL (vincristine, dexamethasone and L-asparaginase, short VXL), act through the induction of apoptosis in cancer cells, defects in the apoptotic pathway can confer resistance to those compounds \[7\]. Besides classical chemotherapeutic drugs, apoptosis impairment also plays a role in the resistance to many novel targeted therapies. For instance, BCR/ABL+ leukemias can become resistant to the BCR/ABL inhibitor imatinib by modulating the pro- and anti-apoptotic balance \[8\]. Since the overexpression of anti-apoptotic factors is a common resistance mechanism for many drugs in various malignancies, numerous efforts have been directed to the development of drugs that can restore apoptotic cell death. This is the case of the small molecule BH3 mimetics, which target one or more anti-apoptotic proteins, and have been shown to have strong activity against many cancer entities, especially hematologic malignancies \[8-11\]. Other drugs designed to restore the apoptotic response include the inhibitors and the inhibitors of the IAPs (inhibitor of apoptosis proteins) called SMAC (second mitochondrial activator of caspases) mimetics (SM) \[12\].

However, as a general strategy, directly targeting the defects in the apoptotic machinery to reactivate apoptotic cell death is potentially problematic. Given the polyclonality of many cancers, it is likely that different clones dysregulate apoptosis through different mechanisms. This renders several targets responsible for the drug resistance \[6\]. We and others hypothesize that simultaneously activating alternative cell death pathways independent of apoptosis may be an effective approach to target and overcome resistance.

Necroptosis, or programmed necrosis, is a form of regulated cell death independent on the apoptotic machinery. Its activation may thus represent a promising alternative to induce cell death in resistant cancer cells. Necroptotic cells exhibit specific morphological features such as increase of the cellular volume, swelling of the mitochondria and rupture of the plasma membrane \[1, 13\]. Mechanistically, necroptosis can be triggered by various stimuli including ligation of death receptors such as TNFR1. After ligation of TNFR1, the activation of necroptosis depends on the cellular context and has been mainly studied in conditions of experimental apoptosis blockade \[1, 14, 15\]. After TNFR1 activation, multiple proteins are recruited to its intracellular domain, including RIP1, a central player in the control of cell survival, apoptosis and necroptosis. The switch of RIP1 from a pro-survival to a pro-death function is controlled, among others, through its ubiquitination by cIAPs, which ubiquitinate RIP1 to maintain its pro-survival state \[16-18\]. Upon deubiquitination, RIP1 can form a cytosolic complex with RIP3, FADD (Fas associated death domain) and caspase-8 and initiate apoptosis through caspase-8 activation or necroptosis through RIP1 and RIP3 phosphorylation. For the final execution of necroptosis, RIP3 phosphorylates MLKL (mixed lineage kinase-domain like) which translocates to and disrupts the plasma membrane \[19-21\]. The molecular mechanisms of necroptosis have been reviewed in detail elsewhere \[1, 15, 16\].

Since necroptosis can induce cell death bypassing the apoptosis blockade, various efforts are being directed to the development of necroptosis-inducing compounds for the treatment of drug resistant cancer. One of such compounds is the small molecule obatoclax (GX15-070). We previously described that obatoclax sensitizes ALL cell lines and refractory patient-derived cells to dexamethasone \[17\]. Its activation may thus represent a promising alternative to induce cell death in resistant cancer cells. Necroptotic cells exhibit specific morphological features such as increase of the cellular volume, swelling of the mitochondria and rupture of the plasma membrane \[1, 13\]. Mechanistically, necroptosis can be triggered by various stimuli including ligation of death receptors such as TNFR1. After ligation of TNFR1, the activation of necroptosis depends on the cellular context and has been mainly studied in conditions of experimental apoptosis blockade \[1, 14, 15\]. After TNFR1 activation, multiple proteins are recruited to its intracellular domain, including RIP1, a central player in the control of cell survival, apoptosis and necroptosis. The switch of RIP1 from a pro-survival to a pro-death function is controlled, among others, through its ubiquitination by cIAPs, which ubiquitinate RIP1 to maintain its pro-survival state \[16-18\]. Upon deubiquitination, RIP1 can form a cytosolic complex with RIP3, FADD (Fas associated death domain) and caspase-8 and initiate apoptosis through caspase-8 activation or necroptosis through RIP1 and RIP3 phosphorylation. For the final execution of necroptosis, RIP3 phosphorylates MLKL (mixed lineage kinase-domain like) which translocates to and disrupts the plasma membrane \[19-21\]. The molecular mechanisms of necroptosis have been reviewed in detail elsewhere \[1, 15, 16\].
We assessed the single agent activity of the SM birinapant and LCL161 both in vitro and in vivo in a broad range of patient-derived pediatric B- and T-ALL samples with a special emphasis on refractory and relapse cases [37]. Birinapant potently induced cell death in around 30% of B-ALL cases at concentrations in the low nanomolar range and was less potent against T-ALL. The response to LCL161, which has been found by others to induce insufficient responses in B-ALL [38], was remarkably less potent in vitro and ineffective in vivo. The structural differences between birinapant and LCL161 might account for the observed differences in activity. Other groups have shown similar promising results for SM in other subtypes of leukemia. Richmond et al. have shown that Philadelphia-like ALL samples have a particularly high sensitivity to birinapant compared to other subtypes of ALL. Furthermore, birinapant enhanced the response of patient-derived samples in vitro to the clinical chemotherapeutic regimen VXL [39]. Similarly, Brumatti et al. show that birinapant effectively kills primary cells harboring the MLL (myeloid/lymphoid or mixed-lineage leukemia 1) translocation in AML mouse models and patient-derived AML samples at concentrations in the nanomolar range [40]. A different SM, BV6, has also been shown to be effective against primary MLL [41].

We used lentCRISPR (clustered regularly interspaced short palindromic repeats) technology to show that the activity of birinapant is completely dependent on RIP1 in vitro and in vivo. Patient-derived ALL cells were transduced with lentCRISPR targeting RIP1 and carrying the fluorescent marker EGFP. We then xenotransplanted these ALL cells into immunodeficient NSG (NOD scid IL2 receptor gamma null) mice and monitored the expansion of WT (EGFP-negative) and knockout (EGFP-positive) cells under treatment with birinapant. We observed a strong selection of the RIP1 deficient cells in vitro and confirmed also in vitro their resistance to SM. The loss of RIP1 did not affect the engraftment kinetics of patient-derived B-ALL in vivo [37].

To further investigate the downstream cell death mechanism induced by birinapant in this context, we developed a multicolor lentCRISPR approach to target multiple cell death genes at the same time. We simultaneously transduced patient-derived cells with lentCRISPR targeting RIP3, caspase-8, FADD or MLKL, each expressing a different fluorescent marker, which allowed us to monitor the expansion of single, double, triple and quadruple knockout cells under birinapant treatment in vivo. Knockout of both an apoptotic (FADD or caspase-8) and a necroptotic (RIP3 or MLKL) gene was necessary to rescue cell viability, and targeting either pathway alone was not sufficient to achieve resistance. In contrast with many studies showing that caspase inhibition is necessary to induce necroptosis, we found that apoptosis and necroptosis were simultaneously activated in around 40% of birinapant-sensitive B-ALL patient-derived samples and in the Jurkat cell line [37]. However, this mixed cell death phenotype was not observed in response to birinapant treatment in AML, where the co-treatment with the pan-caspase inhibitor Z-VAD or the clinical caspase-8 inhibitor emricasan actually increased the response to birinapant by TNFR1-dependent necroptosis. Interestingly, the cell death induced by birinapant+ZVAD in that context was not rescued by neither RIP3 nor MLKL deficiency [40]. This together with our observation that around 20% of birinapant-sensitive B-ALL cases are not rescued by the combined inhibition of apoptosis and necroptosis indicate that other uncharacterized cell death mechanisms besides necroptosis and apoptosis might be activated by RIP1 after SM treatment.

Considering that the current clinical challenge in the treatment of ALL is the appearance of drug resistance and relapse, we wanted to evaluate if birinapant would still be active against heavily pre-treated refractory and relapsed samples. To answer this question we screened CRISPR-generated RIP1 knockout patient-derived B-ALL cells for their response to a panel of relevant anti-leukemic drugs such as vincristine, dexamethasone or doxorubicin among others, indicating that there would be no mutational pressure on RIP1 which could be selected for during the treatment in refractory and relapse patients. Additionally, our initial drug screen included 11 samples from relapse cases, 6 out of which showed IC50s lower than 500nM [37]. Similar conclusions were derived from the studies of Richmond et al., who observed that birinapant was still effective in reducing the leukemic burden in vivo in patient-derived xenografts that had relapsed after pre-treatment with birinapant or VXL [39].

In contrast to the potent activity observed in vitro and in vivo against hematologic malignancies, no anti-tumor activity of birinapant as a single agent could be detected in the phase 2 clinical trial for platinum-resistant and -refractory ovarian cancer [42]. Single agent activity of SM seems to be cancer dependent, with hematologic malignancies (PKC412) [34], and with p38 or MK2 inhibitors in AML (acute myeloid leukemia) [35]. Although generally more effective in combination, some SM such as BV6, LCL161 and birinapant have been described to also induce cell death as single agents in various cancer cell lines including various subtypes of leukemia [30, 32, 34, 36].
representing a subgroup with enhanced sensitivity. Therefore, establishing molecular biomarkers that predict a response to SM is of great importance and many scientists are pursuing this goal. In many cases, especially in the context of cell lines, the production of TNFα in the basal state or after treatment with SM has been shown to correlate with or be necessary for the induction of cell death. In a similar direction, the response to SM has been shown to be dependent on TNFR1 or the TNFR1 signaling pathway, since either treatment with TNFR1 blocking antibodies or TNFR1 knockout confer resistance to SM, and its expression has been found to be higher in SM sensitive cases [40, 41]. However, we and others have found that either TNFα blockade or TNFR1 targeting were not sufficient to rescue from SM-induced cell death [29, 33, 37, 43, 44]. Furthermore, most of the patient-derived B-ALL samples that we tested had very low expression of TNFα both before and after treatment, with no differences between birinapant-responders and -non responders. Other biomarkers of response to SM have been proposed, including cellular levels and activity of caspases-8 and -10 [45, 46], the ability to form the ripoptosome in response to treatment [47] or cIAP2 upregulation or stabilization [48, 49].

In conclusion, to achieve success in the treatment of cancer, resistance to cell death has to be avoided or overcome. Directly targeting the causes of apoptosis blockade or inducing alternative mechanisms of cell death such as necroptosis are two attractive strategies that have been developed to target such resistance, and SM are compounds which can do both. Our data suggest that the SM birinapant has clinical potential for the treatment of refractory and relapsed ALL by inducing apoptosis and necroptosis in parallel. Simultaneously activating two cell death mechanisms, either by a single compound or by a

Figure 1. SMAC mimetics induce concurrent apoptosis and necroptosis in precursor B-ALL and are therefore active against apoptosis-resistant cells.
combination, may be beneficial for cancer therapy by impairing the appearance of resistance mechanisms (Figure 1). Clearly, a deeper understanding of the molecular mechanisms that govern cell death responses, such as necroptosis, as well as the identification of biomarkers that indicate activation of the pathway will be required to move clinical application of compounds such as SMAC mimetics forward.

Conflicting interests

The authors have declared that no conflict of interests exist.

Acknowledgement

The authors thank Albert Bonmassip for help with the figure and Brice Mouttet for comments on the manuscript. This work was supported by the “Stiftung Kinderkrebsforschung Schweiz” and the MAM-Fonds of the Children’s Research Centre of the University Children’s Hospital Zurich.

Author Contributions

J.A.G and B.C.B. jointly wrote the manuscript. Both authors read and approved the final version of the manuscript.

Abbreviations

ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; BCL-2: B-cell lymphoma 2; BH3: BCL-2 homolog domain 3; BCR/ABL: breakpoint cluster region/Abelson; CRISPR: clustered regularly interspaced short palindromic repeats; DISC: death inducing signaling complex; EGFP: enhanced green fluorescent protein; FADD: Fas associated death domain; FLT3: fms like tyrosine kinase; IAP: inhibitor of apoptosis proteins; IL2: interleukin 2; MK2: mitogen-activated proteins kinase-2; MLKL: mixed lineage kinase-like; MOMP: mitochondrial outer membrane permeabilization; Nec: necrostatin-1; NSG: NOD/Scid IL2 receptor gamma null; RIP1: receptor interacting protein kinase 1; RIP3: receptor interacting protein kinase 3; SMAC: second mitochondria-derived activator of caspases; SM: SMAC mimetics; TNFα: tumor necrosis factor α; TNFR1: TNF receptor 1; XIAP: X-linked IAP.

References


CLL cells are resistant to smac mimetics because of an inability to form a ripoptosome complex. Cell Death Dis 2013; 4: e782.
